Beta-carotene and cancer risk : a trial in smokers using biomarkers as intermediate endpoints

Beta-carotene and cancer risk

a trial in smokers using biomarkers as intermediate endpoints

Proefschrift

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Voor Lies en Noud

Contents

Introduction

This thesis describes a number of studies on β-carotene and cancer risk in humans using biomarkers for exposure, susceptibility and biological effects or disease risk. Traditionally, epidemiological studies on cancer risk have focused on clinical outcome on the one hand, and assessment of exposure, often questionnaire-based, on the other. Such studies have been and will be indispensable for the study of human carcinogenesis, but they are as a rule large-scale, costly and lengthy. Moreover, such studies have to rely on extrapolations from animal experimental and *in vitro* work for inferences regarding mechanisms and biological plausibility. In this respect, a promising approach to strengthen epidemiological studies seems to be the application of biomarkers, sometimes referred to as "molecular epidemiology".[1] A biomarker can be described as an indicator on a biochemical, genetic or cellular level reflecting exposure, susceptibility or health status of a subject.[2-5]

The application of biomarkers in epidemiology has received special attention in the field of research on chemoprevention of cancer [4-7] since biomarkers may function as intermediate endpoints or cancer surrogates. Though the predictive value of intermediate endpoints is often not yet established, they can indicate the pathways inhibited and the stages of carcinogenesis affected. Such studies may thus provide valuable information on mechanisms and on biological effectiveness of preventive interventions in humans. They may also be used to select promising agents and/or high-risk groups for full-scale intervention trials on cancer incidence.[5,6]

The studies described in this thesis are focused around an intervention trial to evaluate the effect of β-carotene on several putative markers for cancer risk. The trial was performed in a group of cigarette smokers since they have, in comparison with non-smokers, a high risk for cancer, low plasma levels of β-carotene and increased levels for the biomarkers for cancer risk. The central question in this thesis is whether dietary β-carotene protects against cancer. A literature review (Chapter 2) gives the rationale for performing an intervention trial using β-carotene and summarizes the biological mechanisms by which β-carotene may beneficially influence carcinogenesis. Chapter 3 evaluates the application of biomarkers in a cross-sectional study involving both smokers and non-smokers. For the smokers group, this cross-sectional study provides also the baseline measurement of the intervention trial. The biological significance of one cross-sectional association, between a genetic susceptibility marker and a marker for DNA damage, is addressed in more detail in Chapter 4. The results of the β-carotene intervention trial are described in Chapters 5 to 8. Each of these Chapters addresses the effect of β-carotene on a separate biomarker for early biological effect or cancer risk, and the biological significance of this effect.
The epilogue (Chapter 9) evaluates the knowledge gained from the studies described in this thesis. First, the studies are evaluated in a methodological manner to address their validity for further interpretation. Second, the results of the studies are integrated and discussed in terms

of their contribution to our understanding of the biological mechanisms of carcinogenesis and the involvement of β-carotene in this process. Finally, implications and expectations for future research are discussed.

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Carotenoids and cancer: an update with emphasis on human intervention studies

Geert van Poppel

Abstract

This article gives an overview of the current state of knowledge on the cancer preventive potential of carotenoids. Numerous retrospective and prospective epidemiologic studies have shown that a high intake of carotenoid-rich fruits and vegetables is associated with a decreased risk of cancer at a number of common sites. For several other cancer sites, however, the epidemiological evidence is not very consistent.

A number of mechanisms for the cancer preventive properties of carotenoids have been proposed. Conversion to retinol, possibly in post-hepatic tissues, would allow an effect on cellular differentiation and proliferation, and on cell to cell communication. Antioxidant functions could prevent free radical induced damage to cellular DNA and other macromolecules. Immunomodulatory effects could enhance immune surveillance in tumorigenesis. In addition, non-retinol mediated effects of carotenoids on metabolism of carcinogens and cell to cell communication have been shown.

Observational epidemiology cannot resolve whether associations are due to a specific carotenoid, or to an associated factor in fruits and vegetables, whereas interpretation of animal studies is hampered by uncertainties in extrapolation between species, more so because the metabolism of carotenoids in most animals differs noteably from that in humans. Human intervention studies on biomarkers related to cancer risk and on cancer incidence are therefore necessary. Human intervention studies performed so far suggest that β-carotene can affect carcinogenesis, though not at all stages and not at all cancer sites. Implications for future human intervention research are discussed.

Introduction

In 1981, Peto et al.[1] hypothesized that dietary carotenoids may reduce human cancer rates. Since then, a large number of epidemiologic studies have addressed this topic, and a flurry of experimental work has been aimed at unravelling the possible mechanisms of chemoprevention by carotenoids. This article will give an overview of the current state of knowledge regarding the cancer preventive potential of carotenoids. Firstly, an update on epidemiologic studies regarding carotenoids and cancer will be given. Subsequently, the current concepts on mechanisms of carcinogenesis will be addressed and possible mechanisms of action of

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carotenoids will be discussed. Finally, results of human intervention studies that have so far been performed will be addressed, and implications for future research will be given.

Epidemiologic studies on carotenoids and cancer

A large number of case-control studies have evaluated the association between intake of fruits and vegetables containing carotenoids and cancer. Likewise, prospective cohort studies have evaluated the relation between prediagnostic consumption or blood levels of carotenoids and subsequent risk of cancer. Table 1 and 2 summarize the results for the retrospective and prospective studies.

The results of the case-control studies (table 1) show that high intake of fruits and vegetables that are rich in carotenoids has been associated with decreased risk of cancer at a number of common sites. This association appears to be most consistent for lung cancer and stomach cancer, and least consistent for breast, prostate, esophagal and oral cancer.

Table 1. Retrospective studies on dietary intake of carotenoids and cancer, grouped by site.

First author	no of cases	Exposure measure	Association	Relative risk high vs lowest	Population
Lung					
Maclennan 1977[58]	233	green veg.index	↓	0.45	♂+♀, Singapore Chinese
Kolonel 1983[59]	267	carotenoids	↓	0.40	♂+♀, Multiethnic, Hawaii
Hinds 1984[60]	364	carotenoids	↓	0.45	♂+♀, Multiethnic, Hawaii
Wu 1985[61]	216	β-carotene	↓	0.40	White ♀, LA County
Samet 1985[62]	447	carotenoids	n.s.	0.76	White ♂+♀, New Mexico
Ziegler 1986[63]	763	carotenoids	↓	0.59	White ♂, New Jersey
Pisani 1986[64]	417	carrots/green veg.	↓	0.50	♂+♀, Northern Italy
Bond 1987[65]	308	carotenoids	n.s.	0.42	Chemical Employees, Texas
Byers 1987[66]	450	carotenoids	↓	0.63	♂+♀, New York
Pastorino 1987[67]	47	carotenoids	n.s.	0.34	♀, Northern Italy
Koo 1988[68]	88	carotenoid rich veg.	n.s.	0.60	Chinese ♀, Hong-Kong
Fontham 1988[69]	1253	carotenoids	n.s.	0.88	♂+♀, Southern Louisiana
Le Marchand 1989[70]	332	β-carotene	↓	0.53	♂+♀, Multiethnic, Hawaii
Jain 1990[71]	839	β-carotene	n.s.	0.89	♂+♀, Toronto Area
Dartiques 1990[72]	106	carotenoids	↓	0.25	♂+♀, South-Western France
Harris 1991[73]	96	carotenoids	↓	0.45	♂, United Kingdom
Wu-Williams 1991[74]	965	carotene-rich veg.	n.s.	0.90	♀, North-east China
Esophagus					
Ziegler 1981[75]	120	carotene	n.s.	0.77	Black ♂, Washington DC
Tuyns 1987[76]	743	carotene	↓	0.47	♂+♀, Calvados, France
Decarli 1987[77]	105	β-carotene	↓	0.23	♂+♀, Northern Italy
Brown 1988[78]	209	carotene	n.s.	0.80	♂, South Carolina
Graham 1990[79]	178	carotene	n.s.	0.66	♂+♀, New York

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Table 1, continued

First author	no of cases	Exposure measure	Association	Relative risk high vs lowest	Population
Oral cavity, Pharynx					
Winn 1984[80]	227	fruits & veg.	↓	0.5	Black & white ♀, USA
McLaughin 1988[81]	871	carotene	n.s.	0.9; 0.8	White ♂;♀, USA
Rossing 1989[82]	166	carotenoids	n.s.	1.0	♂+♀, Washington State
Ning 1990[83]	100	carrots	n.s.	0.4	♂+♀, Tianjin, China
Stomach					
Correa 1985[84]	391	carotenoids	n.s.	0.68; 1.08	♂+♀, Louisiana (White; Black)
Risch 1985[85]	246	β-carotene	↓	0.33	♂+♀, Canada
Jedrichowski 1986[86]	110	fruits & veg.	↓	0.24	♂+♀, Cracow, Poland
La Vecchia 1987[87]	206	β-carotene	↓	0.39	♂+♀, Northern Italy
You 1988[88]	564	carotene	↓	0.50	Chinese ♂;♀, Rural Shandong
Pancreas					
Gold 1985[89]	201	raw fruits & veg.	↓	0.55	♂+♀, Baltimore area
Norell 1986[90]	99	vegetables	n.s.	0.5	♂+♀, Sweden
Falk 1988[91]	363	carotenoids	n.s.	0.82; 1.65	♂;♀, Louisiana
Olsen 1989[92]	212	vegetables	n.s.	0.95	White ♂, Minneapolis area
Colon, Rectum					
Macquart-Moulin 1986[93]	399	vegetables	↓	0.42; 0.68	♂;♀, Marseille Region
Potter 1986[94]	419	β-carotene	n.s.	0.8; 2.2	♂;♀, S. Australian
Kune 1987[95]	715	β-carotene	↓	0.45	♂+♀, Melbourne
Graham 1988[96]	428	carotene	n.s.	-	♂+♀, New York
La Vecchia 1988[97]	575	green vegetables	↓	0.5	♂+♀, Northern Italy
West 1989[98]	231	β-carotene	↓	0.4; 0.6	♂;♀, Utah
Freudenheim 1990[99]	423	carotenoids	↓	0.59; 0.70	♂;♀, New York
Peters 1989[100]	147	raw fruits & veg.	↓	0.59	♂+♀, Los Angeles County
Young 1988[101]	353	yellow vegetables	n.s.	0.78	White ♂+♀, Wisconsin
Slattery 1988[102]	231	vegetables	n.s.	0.6	White ♂+♀, Utah
Bidoli 1992[103]	148	vegetables	n.s.	0.6	♂+♀, Northern Italy
Bladder					
Dunham 1968[104]	493	leafy & yellow veg.	n.s.	-	Black & white ♂+♀, Louisiana
Mettlin 1979[105]	569	carrots	↓	0.62	♂ + ♀, New York State
La Vecchia 1989[106]	163	carotenoids	↓	0.41	♂ + ♀, Northern Italy
Risch 1988[107]	826	β-carotene	n.s.	0.95	Canadian ♂ + ♀
Claude 1986[108]	431	fruits & vegetables	↓	0.59; 0.90	♂;♀, Northern Germany
Cervix					
Marshall 1983[109]	513	β-carotene	↓	0.50	White ♀, New York
Brock 1988[110]	117	β-carotene	↓	0.5	♀, Sydney, Australia
La Vecchia 1988[111]	392	β-carotene	n.s.	0.18; 1.09	♀, Italy (Invas;Intraepith)
Verreault 1989[112]	189	carotenoids	n.s.	0.6	♀, Washington State
Ziegler 1990[113]	271	carotenoids	n.s.	0.98	White ♀, USA
VanEenwijk 1991[114]	102	β-carotene	n.s.	0.56	♀, Chicago, USA

continued overleaf

Table 1, continued

First author	no of cases	Exposure measure	Association	Relative risk high vs lowest	Population
Breast					
La Vecchia 1987[115]	1108	green vegetables	↓	0.42	♀, Northern Italy
Iscovich 1989[116]	150	β-carotene	n.s.	0.92	♀, Argentina
Katsouyanni 1988[117]	120	carotene	↓	0.56	♀, Greece
Rohan 1988[118]	451	β-carotene	↓	0.76	♀, South Australia
Marubini 1988[119]	214	β-carotene	n.s.	1.20	♀, Northern Italy
Toniolo 1989[120]	250	β-carotene	n.s.	1.00	♀, Northern Italy
Van 't Veer 1990[121]	133	β-carotene	n.s.	0.73	♀, Netherlands
Potischman 1990[122]	83	carotenoids	n.s.	0.81	♀, New York State
Richardson 1991[123]	409	β-carotene	n.s.	1.0	♀, Montpellier, France
Ovary					
Slattery 1989[124]	85	β-carotene	↓	0.50	White ♀, America
Byers 1983[125]	274	carotenoids	n.s.	0.77	♀, New York
La Vecchia 1987[126]	455	carotene	n.s.	0.94	♀, Northern Italy
Shu 1989[127]	172	carotene	n.s.	1.0	♀, Shanghai, China
Prostate					
Ohno 1988[128]	100	β-carotene	↓	0.34	♂, Japan
Ross 1987[129]	142	β-carotene	n.s.	0.6; 1.0	♂ (black; whites), California
Mishina 1985[130]	100	gr. & yel. veg.	n.s.	0.5	♂, Japan
Talamini 1986[131]	166	green vegetables	n.s.	1.20	♂, Pordenone, Italy
Mettlin 1989[132]	371	β-carotene	↓	0.60	♂, New York
Oishi 1988[133]	100	β-carotene	↓	0.47	♂, Japan
Le Marchand 1991[134]	452	β-carotene	n.s.	-	♂, Multiethic, Hawaii

n.s. = not statistically significant. ♀ = women; ♂ = men.

The studies in table 1 may be hampered by misclassification through a rather crude assessment of dietary intake, especially in the older studies. Presumably, however, this misclassification will be nondifferential and will therefore only attenuate associations. Another caveat in the interpretation of these studies is that dietary information is collected retrospectively from diseased cases. This may potentially introduce differential misclassification since the disease process may have influenced dietary intake, or knowledge of the disease status may result in recall bias in the patient.

Problems of differential misclassification are avoided in prospective studies, since dietary information or blood samples are collected a number of years prior to diagnosis. The results of the prospective studies show a remarkable consistency for the association of increased lung cancer risk with either infrequent consumption of dark green and yellow fruits and vegetables, low levels of dietary carotenoids, or low plasma β-carotene levels. The studies using plasma levels, however, should be considered with some caution since plasma β-carotene levels may be lowered through a metabolic effect of smoking. Though all studies have adjusted their risk estimates for the number of cigarettes smoked, the crude adjustment may not have adequately

accounted for the metabolic effects of smoking on β-carotene.[2] In addition, it should be realized that epidemiological studies based on either dietary intake or plasma levels cannot resolve whether the observed effects are due to dietary carotenoids or to other components of fruits and vegetables.

For stomach cancer, the prospective evidence for a protective effect of consumption of dark green and yellow fruits and vegetables and possibly dietary carotenoids is rather consistent, and in line with the retrospective case control studies, though the magnitude and the number of studies are modest as compared to lung cancer. For breast and prostate cancer, the prospective studies are in line with the retrospective studies and do not indicate a clearly consistent association of plasma or dietary carotenes with reduced cancer risk. For the other cancer sites, the number of cases in the prospective studies are often small, implying that only very strong associations would have been detected in these studies.

Table 2. Prospective studies on dietary intake or blood levels of carotenoids and cancer, grouped by site.

First author	no of cases	Exposure measure	Association	Relative risk high vs lowest	Population
Lung					
Shekelle 1981[135]	33	carotenoid intake	↓	0.14	♂, Western Electric Study
Long-de 1985[136]	2952	green salad + fruit	↓	0.56	♂+♀, China
Hirayama 1986[137]	1917	green & yellow veg.	↓	0.79;1.35	♂;♀, Japan
Kromhout 1987[138]	63	carotenoid intake	n.s.	0.68	♂+♀, Zutphen, NL
Paganini-Hill 1987[139]	56	carotenoid intake	n.s.	0.72;0.67	♂;♀, Leisure World, LA
Knekt 1991[140]	108	carotenoid intake	↓	0.40;0.93	♂, Finland (non-; smokers)
Fraser 1991[141]	61	fruit + green salad	↓	0.26;0.65	White ♂;♀, California Adven.
Willett 1984[142]	17	plasma total carotene	n.s.	-	♂+♀, USA, hypert. follow-up
Connett 1989[143]	66	plasma β-carotene	↓	0.43	♂, USA, MRFIT Study
Nomura 1985[144]	74	plasma β-carotene	↓	0.29	♂, Jap. Ancestry, Hawaii
Wald 1988[145]	50	plasma β-carotene	↓	0.41	♂, UK, BUPA Study
Stähelin 1991[146]	64	plasma total carotene	↓	0.56	♂+♀, Basel Study
Knekt 1990[147]	108	plasma β-carotene	n.s.	1.00	♂+♀, Finland
Comstock 1991[148]	99	plasma β-carotene	↓	0.45	♂+♀, Washington County
Orentreich 1991[149]	123	plasma β-carotene	↓	0.33	♂+♀, USA
Stomach					
Hirayama 1986[137]	5247	green & yellow veg.	↓	0.66;0.66	♂;♀, Japan
Nomura 1985[144]	70	plasma β-carotene	n.s.	-	♂, Jap. Ancestry, Hawaii
Wald 1988[145]	13	plasma β-carotene	n.s.	-	♂, UK, BUPA Study
Knekt 1990[147]	32	plasma β-carotene	n.s.	0.8	♂+♀, Finland
Stähelin 1991[146]	16	plasma total carotene	↓	0.34	♂+♀, Basel Study
Pancreas					
Knekt 1990[147]	10	plasma β-carotene	n.s.	0.60	♂+♀, Finland
Mills 1988[150]	40	green veg. & salad	n.s.	-	♂+♀, California Adventists
Comstock 1991[148]	22	plasma β-carotene	n.s.	0.83	♂+♀, Washington County

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Table 2, continued

First author	no of cases	Exposure measure	Association	Relative risk high vs lowest	Population
Colon, Rectum					
Heilbrun 1989[151]	162	dietary β-carotene	n.s.	0.72	-
Paganini-Hill 1987[139]	110	dietary carotenoids	n.s.	0.90;1.17	♂;♀, Leisure World LA
Shekelle 1981[135]	49	dietary carotenoids	n.s.	-	♂, Western Electric Study
Comstock 1991[148]	106	plasma β-carotene	n.s.	0.83;1.25	♂+♀,Washington,(colon;rectum)
Connett 1989[143]	14	plasma β-carotene	n.s.	-	♂, USA, MRFIT Study
Nomura 1985[144]	113	plasma β-carotene	n.s.	-	♂, Jap. Ancestry, Hawaii
Schober 1987[152]	72	plasma β-carotene	n.s.	0.83	White ♂+♀, Washington
Wald 1988[145]	30	plasma β-carotene	n.s.	-	♂, UK, BUPA Study
Knekt 1990[147]	13	plasma β-carotene	n.s.	0.30	♂+♀, Finland
Stähelin 1991[146]	32	plasma total carotene	n.s.	0.76	♂+♀, Basel Study
Bladder					
Knekt 1990[147]	18	plasma β-carotene	n.s.	3.30	♂+♀, Finland
Paganini-Hill 1987[139]	59	dietary carotenoids	↓	0.62;0.15	♂;♀, Leisure World LA
Shekelle 1981[135]	19	diatary carotenoids	n.s.	-	♂, Western Electric Study
Nomura 1985[144]	27	plasma β-carotene	n.s.	-	♂, Jap. Ancestry, Hawaii
Wald 1988[145]	15	plasma β-carotene	n.s.	-	♂, UK, BUPA Study
Comstock 1991[148]	35	plasma β-carotene	n.s.	0.63	♂+♀, Washington County
Breast					
Hirayama 1979[153]	142	green & yellow veg.	n.s.	-	♀, Japan
Paganini-Hill 1987[139]	123	dietary carotenoids	n.s.	0.83	♂+♀, Leisure World LA
Comstock 1991[148]	30	plasma β-carotene	n.s.	0.9	♀, postmenop. Washington
Wald 1984[145]	39	plasma β-carotene	n.s.	0.35	♀, Guernsey, UK
Willett 1984[142]	14	plasma total carotene	n.s.	-	♀, USA, hypert. follow-up
Prostate					
Hirayama 1986[137]	183	green & yellow veg.	n.s.	1.23	♂, Japan
Mills 1988[154]	180	fruits & salads	↓	0.70;0.68	♂, Calif.Adv. (fruits;salads)
Snowdown 1984[155]	247	fruits & vegetables	n.s.	-	♂, Seventh-day Adventists
Paganini-Hill 1987[139]	93	dietary carotenoids	n.s.	0.98	♂, Leisure World LA
Shekelle 1981[135]	29	dietary carotenoids	n.s.	-	♂, Western Electric Study
Comstock 1991[148]	103	plasma β-carotene	n.s.	0.91	♂, Washington County
Willett 1984[142]	11	plasma total carotene	n.s.	-	♂, USA, hypert. follow-up
Knekt 1990[147]	32	plasma β-carotene	↓	0.20	♂, Finland
Skin					
Shekelle 1981[135]	36	dietary carotenoids	n.s.	-	♂, USA, W.Electric Study
Wald 1988[145]	56	plasma β-carotene	n.s.	-	♂, UK, BUPA Study
Knekt 1991[147]	38	plasma β-carotene	n.s.	3.1	♂+♀, Finland

n.s. = not statistically significant. ♀ = women; ♂ = men.

Current concepts of carcinogenesis

The traditional two-stage view of carcinogenesis, initiation followed by promotion, is derived primarily from animal models on chemical carcinogenesis. Tumor initiation involves exposure of normal cells to chemical, physical or microbial carcinogens that cause a genetic change. The altered genotype of the initiated cell is considered irreversible, but the initiated phenotype is not fully expressed except in the presence of a promotor. Promotors both enhance the expression of the initiated cell phenotype and provide a selective growth stimulus to cells expressing this phenotype, allowing clonal expansion of the initiated cells. This second stage of carcinogenesis, tumor promotion, does not involve a genetic change and is considered reversible.[3]

The classical view of two-stage carcinogenesis involving a mutation (initiation) and an epigenetic change (promotion) has been conceptually important but is also considered to be simplistic in that the number of independent genetic and epigenetic changes may be six or more in certain types of cancer.[4] Carcinogenesis is thus considered as a multistage process; subsequent genetic and epigenetic changes allow susceptible cells to gain a growth advantage and undergo clonal expansions.[4] This probably involves activation of protooncogenes and/or inactivation of tumor suppressor genes (for a review, see reference 5).

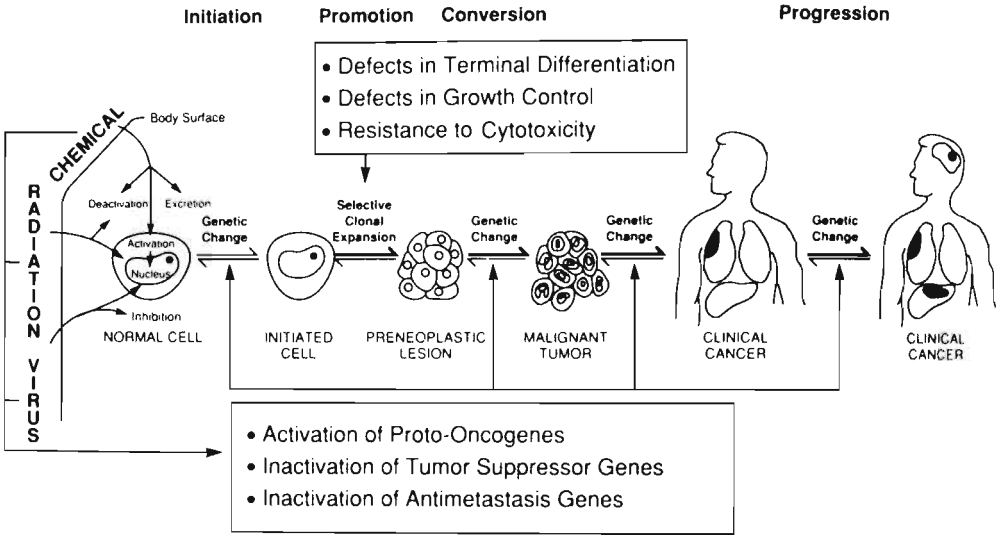

Figure 1. A multi stage model for carcinogenesis involving multiple genetic and epigenetic events (source; ref 4).

A simplified scheme of current concepts of carcinogenesis is given in figure 1. Genetic changes are considered to result from interactions between carcinogen and DNA. Many chemical carcinogens require metabolic activation, generally into high energy electrophiles,

to exert their carcinogenic effect, which is often considered to be mediated through carcinogen-DNA adduct formation.[4] Metabolic activities and genetic damage is assumed to occur within a few hours of exposure.[6] If repair of the damage does not occur within a period of days or weeks, the damage is converted to a stable biological lesion during DNA replication.[6] Tumor promotion enhances the probability of accumulative additional genetic damage including endogenous mutations by allowing expansion of the population of initiated cells. The probability of converting to malignancy may be substantially increased by further exposure to DNA-damaging agents.[4] The conversion of an initiated cel to a premalignant or fully malignant cell is a lengthy process and may take well over 10 years.[6]

Possible cancer preventive activities of carotenoids

Almost 600 carotenoids from natural sources have been characterized. Green leafy vegetables and many colored fruits are rich in carotenoids. Dietary carotenoids in humans are absorbed from the intestine and appear in the blood. Human serum contains β-carotene, α-carotene, cryptoxanthin, lycopene and lutein as major components[7] (figure 2).

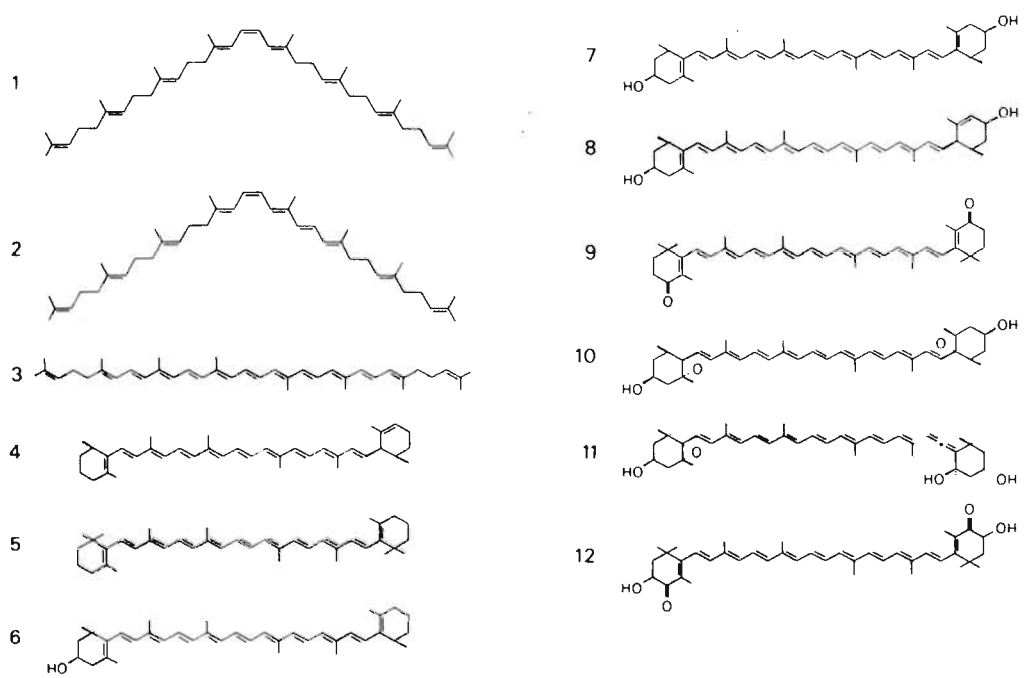

Figure 2. Polyenes and carotenoids in foods that may also be found in animal tissues. 1, phytoene; 2, phytofluene; 3, lycopene; 4, α-carotene; 5, β-carotene; 6, β-cryptoxanthin; 7, zeaxanthin; 8, lutein; 9, canthaxanthin; 10, violaxanthin; 11, neoxanthin; 12, astaxanthin. (source; ref 57).

Carotenoids are present in varying concentrations in adipose tissue, liver, muscle and other tissues, associated mainly with the lipid portions and membranes. A number of biological activities of carotenoids have been described that may pertain to their putative cancer preventive potential:

Conversion to vitamin A

Less than 10% of all carotenoids serve as precursor for vitamin A. In humans, β-carotene, α-carotene and cryptoxanthin are converted to vitamin A, whereas lutein and lycopene are not. The major pathway of conversion is by central cleavage catalyzed by the enzyme 15,15'-β-carotenoid dioxygenase. In addition, random cleavage pathways have been demonstrated in micro-organisms, and fish and birds can also form vitamin A from astaxanthin, canthaxanthin and isozeoxanthin. The enzymatic conversion of carotenoids to retinal is considered to occur almost solely in the intestine and the liver.[8] Blood levels of carotenoids reflect dietary intake, whereas blood levels of retinol are under homeostatic control. Almost all epidemiologic studies that observed reduced cancer risk with increasing β-carotene intake did not observe similar associations with preformed vitamin A intake.[9] The protective action of β-carotene has therefore been considered not to be connected with its conversion to retinol. However, De Vet[10] has argued that epidemiologic studies indicating protection through β-carotene are most consistent for sites involving mainly epidermoid cancers, which is in line with the effects of vitamin A on epithelial differentiation and proliferation. De Vet[10] has therefore hypothesized that β-carotene may exert an influence through local conversion into retinol, i.e. *after* reaching post-hepatic tissues. This possibility is supported by experiments in rats that showed conversion to retinol of intravenously administered β-carotene after removal of the liver, intestine and several other organs.[11-13] Also, recent work by Wang et al.[14] gives evidence for conversion of β-carotene in adipose-, kidney- and lung tissue. The hypothesis of post-hepatic conversion also seems to be supported by recent experiments performed by Edes.[15] He observed that administration of the carcinogen benzo[a]pyrene induced lower tissue levels of retinol in rats. This reduction of retinol could be prevented by administration of β-carotene, but not by retinol administration. If confirmed, these experiments could indicate that β-carotene is capable of rapidly compensating localized vitamin A deficiencies induced by carcinogens.

The mechanisms through which retinoids may influence carcinogenesis have been well documented.[16,17] These mechanisms have been hypothesized to include an action on the cell nucleus, involving the expression of genetic information controlling cell differentiation. Specific binding proteins for retinol and retinoic acid are believed to be responsible for the transport of retinol and retinoic acid within the cell and across the nucleus membrane, suggesting a hormone like control of cell differentiation. In addition, retinol has a variety of effects on the cell membrane, involving altered glycoprotein synthesis and changes in membrane receptors for various hormones, including those mediated by c-AMP. The action on these receptors may influence cell-cell interactions, cell adhesion, and cell membrane permeability. Finally, animal studies have shown that retinol increases both the humoral and

cell-mediated immune response and could thus enhance immune surveillance in tumorigenesis.[18] With regard to the previously discussed model for multi stage carcinogenesis, retinoids thus seem to influence only epigenetic changes, implying an influence primarily in the promotional stages of carcinogenesis. In accordance, an antagonistic effect of retinoids on tumor promotors has been frequently reported in animal studies.[17]

Antioxidant functions

The antioxidant functions of carotenoids are attributed to their molecular structure. This structure would enable them to confer photoprotection by quenching (inactivating) light-energized or excited states of molecules, such as singlet oxygen. Moreover, their structure would enable them to neutralize free radicals, intermediates of metabolism that are highly reactive since they contain a non-paired electron.[19] The photoprotective actions of carotenoids involve a mechanism, in which light energy is transferred from a sensitized or 'excited' molecule to a carotenoid molecule. This reaction yields a non-excited, original sensitizer molecule and a high energy, so called triplet state of the carotenoid. Subsequently, the carotenoid can return to its ground state with the liberation of a small amount of heat. This heat liberation is attributed to rotational and vibrational interactions with the solvent, facilitated by the conjugated polyene nature of the carotenoid.[20] This protective action of carotenoids may be especially important since sensitized molecules can react with biomolecules to generate radical species. Singlet oxygen in particular is an extremely reactive species capable of initiating lipid peroxidation by reactions with polyunsaturated fatty acids, inactivating proteins and enzyme by reactions with amino acids, and damaging RNA and DNA by reactions with guanine.

Carotenoids can not only quench excited species of molecules, but can also react with free radical species. Free radical species can result from photochemical reactions and from oxidant stress e.g. induced by cigarette smoking, but free radicals are also a result of normal cell metabolism.[21] Free radicals from normal cell metabolism include superoxide (O_2^-) as well as the hydroxyl radical (·OH), derived from hydrogen peroxide, and lipid peroxides, e.g. from arachidonic acid metabolism. If the cell is insufficiently protected by enzymatic and non-enzymatic antioxidants, free radicals can react with biomolecules and thus damage cellular structures. This is exemplified by the chain reaction of lipid peroxidation:

Initiation:	LH + ·OH	→	L· + H_2O
Propagation:	L· + O_2	→	LOO·
	LOO· + LH	→	LOOH + L·
Termination:	LOO· + LOO·	→	LOH + L = O + O_2

Enzymatic antioxidants include catalase, superoxide dismutase and the selenium dependent glutathione peroxidase. Non-enzymatic antioxidants include vitamins C and E and

carotenoids.[21] Burton and Ingold[22] have postulated that β-carotene may exert its anti-oxidant function through its ability to interact with a radical (e.g. peroxyl radical) yielding a carbon centered radical species. Since the conjugated bond system of β-carotene would provide considerable resonance stability, this latter radical species would be more stable and have less tendency to react with biomolecules such as lipids (figure 3). However, very little is as yet known about the chemistry of reactions of carotenoids with radicals. β-carotene functions as an antioxidant in many, but not all *in vitro* systems.[23] In this respect, the evidence that β-carotene is a good radical trapping anti-oxidant only at low oxygen partial pressures[22] is of interest, since low oxygen pressures are found in most tissues under physiological conditions.

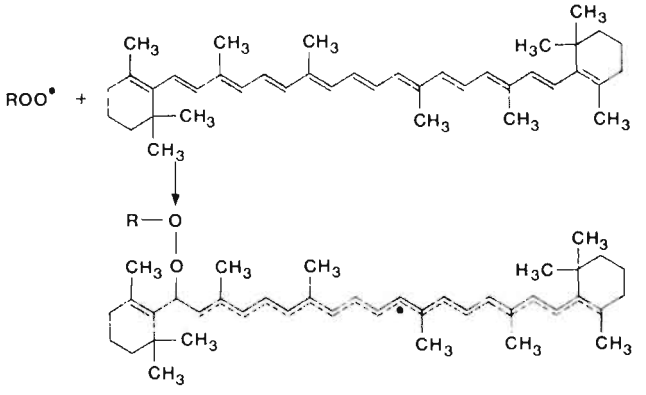

Figure 3. A proposed interaction between β-carotene and a peroxyl radical to produce a resonance-stabilized carbon-centered radical (source; ref 22).

With respect to carcinogenesis, antioxidants have been implied in both the initiation and promotion phases in animal studies.[17] Antioxidants may prevent genetic changes by preventing DNA damage directly induced by free radicals[24] or can hypothetically interfere with the metabolic activation of chemical carcinogens.[25,26] Hypotheses on the role of free radicals and antioxidants in tumor promotion[24,27-29] have been mostly derived from studies using synthetic antioxidants such as BHT or BHA.[17] A number of recent studies have also used carotenoids.[18] Though the molecular mechanisms are not clear, the proposed involvement of antioxidants in tumor promotion stems from observations in animal studies that 1) free radical generating compounds are tumor promotors, 2) well known promotors such as phorbol acetate have been shown to stimulate oxygen radical production, 3) promotors can modulate antioxidant defense mechanisms and 4) antioxidants are antipromotors.

Immunomodulatory effects

Several animal studies have shown that carotenoids may exert immunomodulatory functions. For example, T and B lymphocytes in the spleens of rats showed enhanced proliferative responses after supplementation with eiter β-carotene or cathaxanthin,[30] whereas hamsters with chemically induced tumors showed increased numbers of cytotoxic T cells.[31] The postulated immunomodulatory effects of carotenoids have summarized by Bendich.[31] The mechanisms by which carotenoids may influence immune response are not yet clear. Immuno-enhancing mechanisms have been postulated to involve the quenching of free radicals, which could lower the level of immunosuppressing lipid peroxides, alter arachidonic acid metabolism, stabilize lysozome membranes, or protect nuclear structures. Alternatively, retinol mediated mechanisms could be involved, since retinol has several influences on the immune system.[18]

A possible immunomodulatory effect of carotenoids would imply an action in the promotion phase of carcinogenesis. However, the concept of immunoregulation of carcinogenesis may only apply to certain forms of human cancer, since a substantial number of human cancers does not exhibit any immunogenic properties.[32]

Other mechanisms

In addition to the above mentioned mechanisms other effects of carotenoids have been described. Edes et al.[33] observed modulation of the cytochrome P450 dependent Aryl Hydrocarbon Hydroxylase (AHH) in rat liver by β-carotene, whereas Tan and Chu[34] performed *in vitro* experiments that showed modulation of P450 mediated benzo[a]pyrene metabolism by carotenoids. These observations suggest that carotenoids could modify the enzymatic activation of (pro)carcinogens. Another mechanism of action has recently been suggested by Zhang et al.[35] They observed enhancement of gap-junc communication by several carotenoids in *in vitro* experiments. Enhanced cell to cell communication would restrict clonal expansion of initiated cells and has been described to be brought on by retinoids.[36] In the mentioned experiments however, the effect of carotenoids was not dependent on their pro-vitamin A activity.

Human intervention studies

The possible mechanisms of action for carotenoids given above have mainly been derived from *in vitro* experiments. The interest in carotenoids and cancer has also prompted a number of animal studies, which have recently been reviewed by Krinsky.[23] Many, though certainly not all studies indicate that carotenoids can prevent the appearance of skin tumors induced by ultraviolet light with or without chemical carcinogens. In addition, carotenoids may also prevent internal tumors induced by carcinogens. The results of these studies are not always consistent, and many workers have used systems unique to their own laboratory. Moreover, the metabolism of carotenoids in most laboratory animals differs noteably from that in humans. Intervention studies in humans are therefore necessary to substantiate the evidence from observational epidemiology, *in vitro* experiments and animal studies.

Table 3. Ongoing human intervention trials using β-carotene*.

Target site/organ	Target/high risk group	Inhibitory Agents	Investigator	Location
All sites	physicians	β-carotene	Hennekens	Boston, MA, USA
Colon	colon polyps	β-carotene	Bowen	Illinois, CH, USA
Colon	colon polyps	β-carotene, vit C & E	Greenberg	Hannover, NH, USA
Colon	colon polyps	β-carotene, fiber, low fat	MacLennan	Brisbane, Australia
Lung	asbestosis	β-carotene, retinol	Omenn	Seattle, WA, USA
Lung	smokers	β-carotene, vit E	Huttunen/ Albanes	Helsinki, Finland Bethesda, MD, USA
Lung	tin miners in China	β-carotene, retinol, vit E, Se	Schatzkin	Bethesda, MD, USA
Lung	DNA damage, smokers	β-carotene	Van Poppel	Zeist, Netherlands
Lung	asbestos workers	β-carotene, retinol	Musk	Perth, Australia
Skin	albinos	β-carotene	Luande	Dar es Salaam, Tanzania
Skin	basal cell carcinoma	β-carotene, vit C & E	Safai	New York, USA
Skin	basal carcinoma	β-carotene	Siu	Calgary, Canada
Esophagus	dysplastic patients + high risk group	multiple vitamin + β-carotene	Taylor	Bethesda, MD, USA Beijing, China
Mouth, esophagus	oral leukoplakia + oesophagitis	β-carotene, retinol riboflavin	Zaridze	Uzbekistan, USSR
Mouth	oral leukoplakia	β-carotene	Garewal	Tuscon, AZ, USA
Cervix	cervical dysplasia	β-carotene, vit C, folic acid	Romney	New York, USA

* Adapted from:
- Cullen JW: The National Cancer Institutes Intervention Trials. Cancer 62, 1851-1864, 1988.
- Coleman M, Wahrendorf J (eds). Directory of on-going research in cancer epidemiology 1991. IARC Scientific Publication no. 110. Lyon International Agency for Research on Cancer 1991.

Human intervention studies use either cancer incidence or mortality as an endpoint, or they focus on intermediate endpoints or early biomarkers for cancer risk.[37] These biomarkers are parameters for (alterations in) functions or cellular structures that are thought to bear relevance to carcinogenesis. Such biomarkers include measurements for DNA damage, e.g. micronuclei, measurements of immune system cells or immunological response, or potentially pre-malignant lesion e.g. leukoplakia or dysplasia. For most of these biomarkers, the predictive value for ultimate cancer development has not been unambiguously established. Studies on biomarkers do however have the merit of yielding information on mechanisms of action in humans, whereas they do not imply the long time span necessary for studies on cancer incidence. Moreover, biomarker studies allow for a considerable smaller study size and can

therefore often be better controlled. Biomarker studies can thus yield important information that can be used for both evaluation of cancer preventive potential as well as for optimizing the design of large-scale intervention studies on cancer incidence. Human intervention studies on biomarkers or cancer incidence that are in progress are listed in table 3. Studies that have already published results are discussed below.

Studies on the effect of carotenoids and retinol on micronuclei in buccal mucosa of tobacco chewers have been performed by Stich and co-workers. Micronuclei are DNA fragments in exfoliated cells that occur after carcinogenic exposure. Several lines of evidence indicate that they may provide a marker for early stage carcinogenesis, but their predictive value has not been established. Strong reductions in micronucleated cells were observed after Indian and Fillipino villagers had been supplemented with β-carotene (180 mg/week), β-carotene combined with retinol (100.000 IU/week), or retinol alone.[38-40] Since these villagers were considered to have marginal vitamin A status, these results may be attributable an enhanced vitamin A intake, and not to a specific carotenoid effect; in accordance, canthaxanthin was not effective.[39] However, it is not clear whether cathaxanthin does reach the buccal mucosa, whereas this has indeed been shown for β-carotene.[41,42] A specific carotenoid effect therefore cannot be ruled out, even more so because a subsequent study showed β-carotene to be effective in Inuit snuff users from a population having normal plasma retinol levels.[43] The latter study, however, did not monitor individual plasma retinol or antioxidant levels during the trial.

Immunological studies in humans on the effect of β-carotene so far have focused on assessment of lymphocyte subpopulations in peripheral blood. These studies must be cautiously interpreted, since the relevance of peripheral lymphocyte subpopulations for functional immune response in target tissue in not certain. So far, the studies have yielded inconsistent results. Alexander et al.[44] reported an increase in the number of lymphocytes expressing CD4+ (indicating T helper function) after two weeks of β-carotene (180 mg/day) in normal human volunteers. This study, however, was not placebo controlled, and plasma retinol levels were not monitored. Another study in patients with precancerous lesions[45] used 30 mg/day for three months and reported an increase in percentage of lymphocytes expressing Leu-11b (indicating natural killer cell function) and interleukin 2 receptors, whereas the percentage of cells expressing CD4+ was not affected. This study, again, was not placebo controlled and did not monitor retinol levels. Watson et al.[46] performed a study in 20 healthy subjects aged 50-65 years using β-carotene doses of 0, 15, 30, 45, and 60 mg/day and report increases in the percentage of lymphoid cells expressing CD4+ and interleukin 2 receptor, as well as an increase in cells expressing CD16+ (indicating natural killer cell function). Watsons study did not observe concomitant changes in plasma retinol, but his study has been methodologically criticized.[47] In contrast to Watsons study, Ringer et al.[48] did not observe any effects on lymphocyte subpopulations in a placebo controlled study in 50 healthy males and females, using doses up to 300 mg/day.

The effect of 6 months β-carotene, with or without retinol, was studied in Indian tobacco chewers showing oral leukoplakia. Oral leukoplakia is a potentially pre-malignant lesion in

oral carcinogenesis,[40] but the predictive value has not been established. As compared with a placebo group, the combination of β-carotene (180 mg/week) and vitamin A (100,000 IU/week) resulted in more frequent regression of established leukoplakias, and less frequent appearance of new leukoplakias. β-carotene alone showed some effects, but these were not significant. Here again, the participants may have been vitamin A deficient, and plasma retinol levels of the participants were not monitored. Results by Garewal[49] indicate (partial) regression of oral leukoplakias in 17 of 24 patients after 3-6 months β-carotene (30 mg/day). However, this pilot study was not placebo-controlled, results may be partly explained by regression to the mean, and plasma retinol levels were not monitored. A randomized, placebo controlled, 18 month study on oral leukoplakia is now being conducted by the same group.[50]

De Vet et al.[51] studied the effect of β-carotene (10 mg/day for 3 months) on regression or progression of cervical dysplasia, a putative precursor lesion of cervical carcinoma. Their carefully designed placebo controlled study showed no effect of β-carotene. Possibly, their dose of β-carotene may have been to low to demonstrate short-term effects. Also, the recently available evidence from observational case-control studies does not indicate a consistent inverse relation between cervical cancer and dietary intake of carotenoids (table 1). Finally, β-carotene may not affect the late (promotional) stages of carcinogenesis that are reflected by cervical dysplasia.

So far, only one β-carotene intervention study on cancer incidence has been reported.[52] This 5 year placebo controlled trial showed no effect of β-carotene (50 mg/day) on the occurrence of new skin cancers in persons with previous nonmelanoma skin cancers. It has been argued, however, that an effect of β-carotene in this study on cancer recurrence could only be expected if β-carotene were effective in very late stages of carcinogenesis.[53] Moreover, the only three observational epidemiologic studies performed so far did not show an inverse relation between carotenoids and human skin cancer (table 2).

Conclusions and implications

A number of human intervention studies on cancer or biomarkers related to cancer risk have been performed so far. These studies have all focused on β-carotene because this carotenoid has both pro-vitamin A and anti-oxidant capacity, and has been proven non-toxic.[54] Studies on buccal micronuclei and oral leukoplakia support a cancer preventive role for β-carotene, whereas studies on peripheral immune cells show conflicting results. A study on skin cancer recurrence and a study on cervical dysplasia were negative. Some results thus suggest that β-carotene can affect human carcinogenesis, whereas other results indicate that this will not occur at all stages of carcinogenesis, or at all cancer sites.

It seems advisable to aim future human intervention studies at cancer sites that have been most consistently associated with carotenoid protection in observational studies. Such studies should be methodologically sound, i.e. randomized and placebo-controlled. Moreover, they should monitor the status of retinol. Since several antioxidants may act in a synergistic manner, it also seems warranted to monitor the status of several anti-oxidants in intervention trials. Also, in

studies using biomarkers, application of a relevant set of biomarkers may yield more insight in the mechanisms of action and possible site specificity of carotenoids.

More insight into the putative cancer preventive potential of β-carotene can be expected from two large ongoing randomized trials. The US-Finland lung cancer prevention trial is evaluating the effect of β-carotene (20 mg/day) on lung cancer incidence in 19.500 male smokers.[55] However, the proposed follow up of 6 years may be insufficient if β-carotene is primarily effective in earlier stages of carcinogenesis. A randomized trial in 22.000 US Physicians is evaluating the effect of β-carotene (50 mg on alternate days) on cancer at all sites.[56] This trial has recently been extended to 12.5 years follow up.[53] However, lung cancer incidence may not be very large among predominantly non-smoking physicians.

This overview has shown that a high intake of carotenoid-rich fruits and vegetables is associated with a decreased risk of cancer at a number of common sites in epidemiological studies. For several other sites, however, the evidence is not consistent. This association may indeed be due to carotenoids, and not to associated food factors, since a number of plausible cancer preventive mechanisms for carotenoids have been suggested. It is envisaged that a number of human intervention studies, that are currently being conducted, will provide more answers regarding the proposed cancer preventive properties of particularly β-carotene.

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Markers for cytogenetic damage in smokers: associations with plasma antioxidants and glutathione S-transferase μ

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Abstract

Biomarkers for increased cytogenetic damage in smokers include sister chromatid exchanges (SCE) in peripheral lymphocytes and micronuclei in sputum cells. These markers may reflect increased cancer risk. Increased cancer risk has also been associated with lower blood levels of the antioxidants β-carotene and vitamin C and with genetic deficiency of the detoxification enzyme glutathione S-transferase μ (GST-μ). We therefore evaluated the associations of plasma antioxidants, GST-μ phenotype and indices for tobacco exposure with SCEs and micronuclei in a group of 156 male cigarette smokers and 38 non-smokers.

As expected, smokers as compared with non-smokers had higher SCE levels (5.08 vs. 4.71 SCE per lymphocyte) and lower levels of plasma β-carotene (0.31 vs. 0.48 μmol/l) and blood vitamin C (36.6 vs. 54.8 μmol/l). In smokers, SCEs were weakly correlated with plasma cotinine ($R = 0.186$) but not with plasma antioxidants (all $R < 0.04$). Micronuclei in smokers were not correlated with either cotinine or antioxidants (all $R < 0.14$). As reported previously, SCEs were higher (5.24 vs. 4.97 SCE per lymphocyte) in GST-μ deficient smokers than in nondeficient smokers. Micronuclei, however, were similar in both GST-μ phenotypes (4.3 vs. 4.9 micronuclei/3000 cells). No correlation was observed between micronuclei and SCEs ($R = -0.025$).

Large random variations in both SCEs and micronuclei make it difficult to interpret the absence of relations unambiguously. The results indicate that SCEs and micronuclei have only limited sensitivity to variations in cigarette smoke exposure. The association between GST-μ and cancer risk may be mediated through increases in certain forms of smoking induced DNA damage in GST-μ deficiency.

Introduction

Biomarkers are increasingly used in cancer epidemiology to estimate exposure to carcinogens or putative anticarcinogens, preclinical biological effects, as well as genetic factors that may determine individual susceptibility.[1] Markers for DNA damage are of special interest since DNA damage is a crucial step in carcinogenesis.[2] Markers for DNA damage such as SCE[3] and micronuclei[4] are increased in smokers, who have a known increased risk of cancer.[5]

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Despite a large risk, a majority of smokers do not develop cancer. Smokers may be partially protected by dietary anti-oxidants such as β-carotene and vitamin C.[6,7] Also, genetical differences in detoxification of tobacco constituents may determine individual cancer risk.[8-10] The possible protection against cancer of antioxidants has been hypothesized to involve decreased DNA damage.[11] Likewise, a more efficient detoxification of tobacco smoke could offer protection by limiting DNA damage.[8-10] It can therefore be hypothesized that increased antioxidant status and more successful detoxification will correspond with less DNA damage in smokers.

We have previously used the SCE measure to demonstrate an association between deficiency in the detoxification enzyme GST-μ and increased cytogenetic damage in smokers.[12] We now have new data on blood levels of antioxidants and micronuclei in expectorated sputum from the same cross sectional study. This allowed us to evaluate whether the two biomarkers for cytogenetic damage are inversely related with markers for antioxidant status, and positively associated with markers for cigarette smoke exposure. Also, the association of GST-μ phenotype with micronuclei was studied, and we evaluated whether SCE and micronuclei are associated.

Subjects and methods

Subjects

We studied healthy male volunteers, employed at the AMEV Insurance Company, the Taxation Office and the Power Company at Utrecht. The study was approved by an external Medical Ethical Comity and all participants gave their informed consent. Smokers were studied if they reported consumption of more than 15 cigarettes per day over more than two years. Non-smokers were included in this study if they reported never to have smoked and, in addition, did not work or live together with smokers. None of the participants used vitamin preparations containing retinol or carotenoids, or medications known to influence SCE levels. Moreover, they reported not to be exposed to chemicals during working or leisure time. Initially, 163 smokers and 38 non-smokers volunteered and were eligible for the study. The smokers were studied as part of an intervention trial.[13,14] The present analysis is limited to the 156 smokers and 38 non-smokers for whom SCE data are available. The GST-μ assay was ambiguous for one smoker and missing for another smoker. Sputum samples were only collected by smokers since non-smokers do not spontaneously produce sputum. In the smokers group, 29 participants failed to produce sputum samples whereas insufficient sputum cells could be evaluated in another 7 smokers.

Blood parameters

Non-fasting blood samples were collected between 8.00 am and 12.00 am. Directly after venipuncture blood samples were stored in the dark at 0 to 4°C. After 20 to 23 hours overnight dark storage at 4°C, a separate evacuated tube containing heparin as anticoagulant was opened to determine the sum of ascorbic acid + dehydro-ascorbic acid (vitamin C) in

whole blood by HPLC with fluorometric detection.[15] All-*trans* retinol, α-tocopherol, β-carotene and total carotenoids were assayed in EDTA plasma (stored at −80°C) by HPLC with colorimetric detection.[16] For these assays, coefficients of variation for samples from a plasma pool were 4.3, 4.1, 7.0, and 5.7% respectively. Plasma cotinine levels were determined by gas chromatography.[17] Analysis of blind split samples for both a smoker (17 duplicate samples) and a non smoker (7 duplicate samples) revealed a variation coefficient of 3% for the cotinine assay. Presence or absence of GST-μ was established in heparinized whole blood using an enzyme-linked immunosorbent assay (MUKIT, Medlabs, Dublin, Ireland).

Sister chromatid exchanges

Blood cultures for determination of SCEs in lymphocytes[18] were set up within 2 to 6 hours after venipuncture, after the blood had reached room temperature for 30 minutes. For the total study group, blood cultures were set up on 18 separate days; non smokers were studied on 10 of these 18 days. Heparinized whole blood (0.5 ml) was added to 4.4 ml pre-warmed RPMI 1640 medium (Flow) containing 20% foetal calf serum (inactivated for 30 minutes at 56°C), 2.5% phytohaemaglutinin (HA-15 Welcome), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10 μg/ml 5-bromo,2-deoxyuridine. The blood was cultured in the dark at 37°C in T-25 culture flasks (Costar) in 5% CO_2 for 68 ± 1 hour. Colcemid was added to a final concentration of 0.2 μg/ml for the last two hours of incubation. The cells were collected by centrifugation, treated with hypotonic KCl (0.075 M) for 8 minutes to spread the chromosomes and to haemolyse the RBC, and fixed three times with methanol-acetic acid (3:1). After overnight storage in the dark at 4°C, cells were transferred to microscopic slides and air-dried. Preparations aged for 3 days and were stained by the Fluorescence plus Giemsa Technique[18] to obtain harlequin chromosomes. For each subject SCEs in 50 second-division metaphases were scored as colour changes in the longitudinal direction of the chromatid, excluding the centromere. Only metaphases with 46 chromosomes were scored. Individual data are the mean counts of 50 metaphases. SCEs were scored by a single observer in the non-smokers group, whereas an additional observer assisted in scoring SCEs in the smokers group.

Micronuclei in sputum

Sputum was collected and processed as described in detail by Saccomano et al.[19] Each participant received a careful individual instruction on how to produce a specimen from "deep in the lungs". Sputum was collected at home on three consecutive mornings, directly after rising and after carefully rinsing the mouth. The three, or minimally two samples collected in preservative (50 ml 50% ethanol with 2% polyethylene glycol (Carbowax 1540, Merck) were mixed, homogenized, centrifuged and smeared onto slides. Sputum was collected on the days following the venipuncture. The slides were stained with Feulgen and fast green, which is specific for DNA and strongly highlights micronuclei.[20] For each subject, 3000 cells were examined and evaluated on the basis of the following criteria: shape and size typical of epithelial cells, a well defined nucleus and a clearly defined cytoplasm. The criteria in defining a micronucleus were: chromatin structure and colour intensity similar to those of the main

nucleus; on focusing, the micronucleus must be on the same level as the nucleus, must be roundish and clearly included in the cytoplasm. The dimensions should be less than 1/5 of that of the main nucleus, and it should not be connected to it. Slides were screened at 400x magnification and micronucleated cells were examined at 1000x magnification. Slides were read coded/blinded by a single observer. Repeated blinded scoring of 9 samples yielded a good correlation (Pearson R = 0.86), with 2 of the 9 samples showing a difference of more than 1 micronucleus upon rescoring.

Data analysis

Smoking and non-smoking groups, as well as GST-μ deficient and non-deficient groups were compared using the Student t-test and the chi-square test. Associations between variables were evaluated using simple and multiple linear regression. For micronuclei counts, square roots of observations were taken to stabilize variances before data analysis. In addition to the linear regression techniques, the untransformed micronuclei data were also analyzed using Poisson regression. Data analysis were performed using the BMDP package.[21] For the Poisson regression, the GENSTAT program was used.[22]

Results

Table 1 shows the data for the smoking and non-smoking group. Plasma cotinine levels reflect the large contrast in cigarette consumption between both groups. Age, body mass index and

Table 1. Characteristics and blood parameters in smokers and non-smokers.

	Smokers (n = 156)	Non-smokers (n = 38)
Age	39.0 ± 9.6	37.0 ± 10.5
Body Mass Index (kg/m^2)	24.5 ± 3.0	23.6 ± 2.4
Alcohol (g/day)*#	12.1 ± 13.1	2.8 ± 4.2
Cigarettes/day	21.1 ± 6.5	0
Duration of smoking (yrs)	20.9 ± 10.0	0
Plasma cotinine*	327.7 ± 117.1	0.6 ± 0.5
Blood vitamin C*	36.6 ± 17.9	54.8 ± 11.5
Plasma retinol	2.31 ± 0.54	2.16 ± 0.38
Plasma α-tocopherol*	30.9 ± 6.5	27.3 ± 7.0
Plasma β-carotene*	0.31 ± 0.18	0.48 ± 0.30
Plasma total carotenoids*	1.56 ± 0.59	1.91 ± 0.72
SCE per lymphocyte*	5.08 ± 0.98	4.71 ± 0.35
Micronuclei per 3000 sputum cells	4.6 ± 3.7	-

* Smokers different from non-smokers; $p < 0.01$.

\# data for 143 smokers and 35 non-smokers.

plasma retinol are similar in both groups, though slightly higher in the smoking group. In the smoking group, there are more alcohol users (78 vs. 63%) and the mean alcohol consumption is substantially higher. All plasma antioxidants are higher in non-smokers than in smokers, except for plasma α-tocopherol which is slightly though significantly higher in smokers. The SCE measure is about 10% increased in smokers. Adjustment for alcohol consumption did not alter this difference (SCE = 0.37 before adjustment, 0.33 after adjustment). The distribution of SCEs is given in figure 1, whereas the distribution of micronuclei counts is given in figure 2.

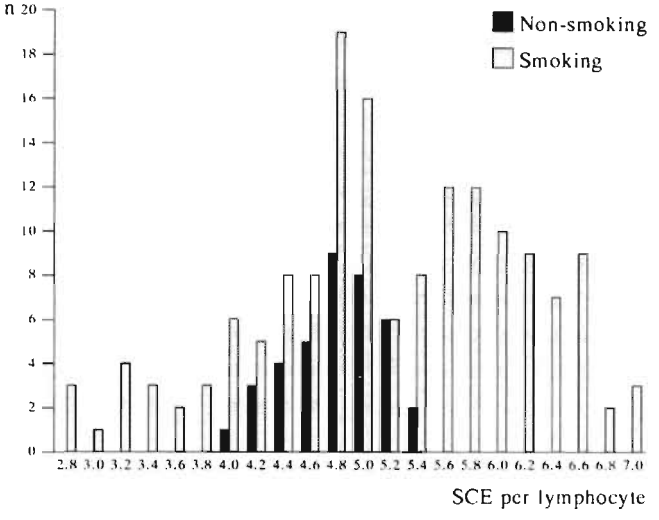

Figure 1. Distribution of sister chromatid exchanges in smokers (n = 156) and non-smokers (n = 38). Numbers on abscissa, upper boundaires of categories for SCEs.

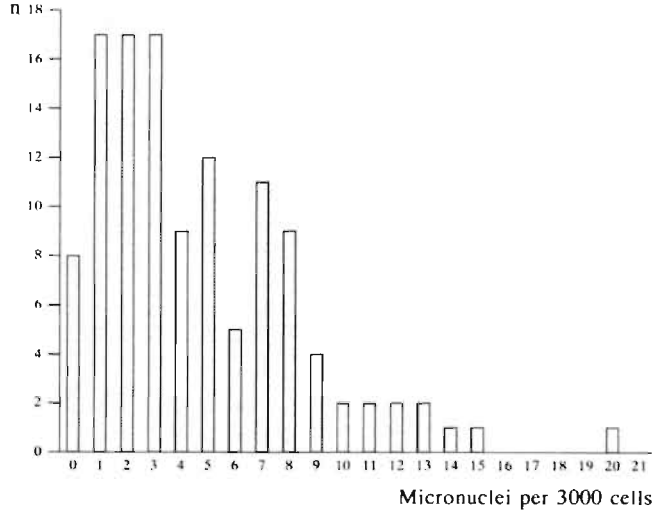

Figure 2. Distribution of sputum micronuclei counts in smokers (n = 143).

The correlations for SCEs, micronuclei, and plasma cotinine for the smokers group are given in table 2. Plasma cotinine is clearly positively associated with the reported cigarette consumption and weakly though significantly with age, smoking years, and, unexpectedly, plasma β-carotene. SCE levels show a weak significant association with both plasma cotinine and reported cigarette consumption, but no association whatsoever with plasma antioxidants or alcohol consumption. No association was observed between SCEs and micronuclei in the smokers group (R = –0.025; figure 3). For the non-smokers group, there were no significant associations of SCEs with any of the parameters in table 2 (all R < 0.14). Micronuclei counts were not associated with any of the indices for tobacco consumption or antioxidant status. Poisson regression models for the micronuclei counts yielded results similar to those in table 2: none of the associations with the listed parameters was significant.

Table 2. Correlation (Pearson coefficients) of several characteristics and blood parameters with SCE, micronuclei and plasma cotinine in male cigarette smokers (n = 156).

	Plasma cotinine	SCE	Micronuclei (n = 120)
Age	0.193*	0.152	0.054
Body Mass Index (kg/m²)	–0.163*	0.083	0.006
Alcohol (g/day)	–0.085	0.052	–0.057
Cigarettes/day	0.368*	0.248*	–0.006
Duration of smoking (yrs)	0.239*	0.109	0.006
Plasma cotinine	1.000	0.186*	–0.013
Blood vitamin C	0.042	0.039	–0.115
Plasma retinol	0.058	0.063	0.069
Plasma α-tocopherol	0.059	–0.022	0.007
Plasma β-carotene	0.177*	–0.038	0.109
Plasma total carotenoids	–0.090	–0.021	0.136

* $p < 0.05$.

Table 3. SCEs and micronuclei in GST-μ-positive and GST-μ negative non-smokers, smokers, 'light' smokers (plasma cotinine < 315 ng/ml) and 'heavy' smokers (plasma cotinine > 315 ng/ml).

		GST-μ negative	GST-μ positive
Non-smokers	SCEs	4.69 ± 0.35	4.74 ± 0.35
	Micronuclei	–	–
Smokers	SCEs#	5.24 ± 0.95	4.97 ± 0.98
	Micronuclei	4.3 ± 4.3	4.9 ± 2.9
Light smokers	SCEs	4.95 ± 0.99	4.97 ± 1.01
	Micronuclei	4.1 ± 3.0	4.9 ± 3.0
Heavy smokers	SCEs*	5.50 ± 0.84	4.97 ± 0.97
	Micronuclei	4.5 ± 5.0	5.0 ± 2.9

* different $\mu-$ from $\mu+$, $p = 0.01$.

p value for comparison $\mu-$ vs. $\mu+$ = 0.09.

Of all subjects, 45% were deficient in GST-μ. We observed no association between GST-μ and any of the blood parameters or characteristics given in table 2. The association of GST-μ deficiency with SCEs and micronuclei is given in table 3. In non-smokers, there is no relation between GST-μ deficiency and SCE levels. In GST-μ deficient smokers, SCE levels are higher than in non-deficient smokers. This μ-related difference is more pronounced in 'heavy' smokers (plasma cotinine above the median), whereas it is absent in 'light' smokers. In the heavy smokers, the μ-related difference was similar in multiple regression analysis controlling for age, body mass index, duration and quantity of smoking, and cotinine levels (5.47 vs. 4.99; $p = 0.02$). In the light smokers, the multivariate adjustment also yielded similar results (4.94 vs. 4.98; $p = 0.87$). Similar analyses for micronuclei in smokers did not reveal any associations with GST-μ phenotype, either in light or heavy smokers (table 3).

For the SCE count in smokers, we observed an effect of culturing day variation (multiple partial $R = 0.53$, $p < 0.001$) and variation between observers (partial $R = 0.51$, $p < 0.001$) in a model that included 18 runs and 2 observers (multiple $R = 0.71$ for the total model). Adjustment for culturing day and observer variation did not materially alter the SCE data and correlations for SCEs given in the tables 1-3. Only the difference in SCEs between smokers and non-smokers was more pronounced after this adjustment (4.85 vs. 5.66 SCE per lymphocyte; $p < 0.001$). For the determination of micronuclei, we did not observe a significant effect of the 18 different runs on the micronuclei counts (multiple $R = 0.42$, $p = 0.20$ in linear regression, $p = 0.20$ in multiple Poisson regression).

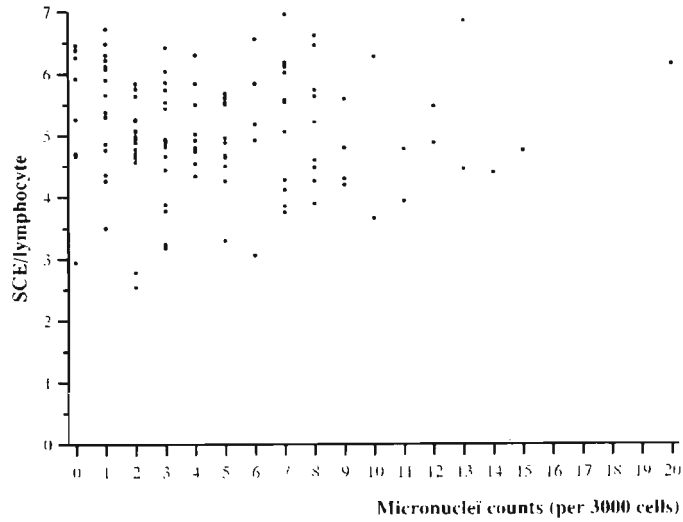

Figure 3. Relationship between sputum micronuclei counts and lymphocyte SCE in smokers (n = 143).

Discussion

The decreased blood levels of carotenoids and vitamin C that we observe in smokers are in line with previous studies.[23,24] These differences may reflect a decreased dietary intake of vitamin C and carotenoids in smokers or a metabolic consequence of cigarette smoking.[23,24] A metabolic effect is biologically plausible, since cigarette smoke is a major source of free radicals and oxidant stress.[25] Our study did not measure dietary intake, but a metabolic effect of smoking is not clearly supported by the low correlations that we observe between plasma levels of cotinine, vitamin C, and carotenoids. For plasma cotinine and plasma β-carotene, we even observe an unexpected weak positive association. The antioxidant vitamin E is slightly, although significantly, higher in smokers than in non-smokers, but this may be explained by higher plasma lipid levels, which are common in smokers.[26] We had no lipid measurements to standardize the vitamin E levels.[27]

An increase in SCE levels in smokers is also a well known phenomenon, with levels being 10-88% higher than in non-smokers.[5] It should be noted that SCE levels observed in several studies cannot readily be compared, since laboratory protocols are not standardized.[18] The sensitivity of the SCE determination to laboratory variations is illustrated by the significant variation over the 18 culturing days, which we observe despite adherence to a strict protocol. Non-laboratory-related differences between the culturing days may also have attributed to this variation. In the analyses within the group of smokers (tables 2 & 3, figure 3) the scorer- and run-variations did not bias our results, since the smokers were randomly divided over the scoring runs, and the observers were equally represented in every scoring run. The analyses adjusted for run- and observer-variation therefore yielded results similar to the unadjusted analyses. The potential for bias, however, is illustrated by the contrast between non-smokers and smokers. This contrast increased after adjustment for run- and scorer-variation, reflecting the unequal distribution of non-smokers over the runs and scorers. This is also exemplified by figure 1, showing that the overall variation in SCE counts is far less in the non-smoker group than in the smoker group.

The correlations within the group of smokers between reported cigarette consumption or plasma cotinine are rather small. Reported cigarette consumption may not be a good measure of genotoxic exposure due to differences in brands and smoking and inhaling practices.[28] Also, the relation between nicotine content and content of genotoxic agents in cigarettes is uncertain. However, since we also observe an only 10-20% increase in smokers as compared with non-smokers, the conclusion that the SCE measure in lymphocytes is not very sensitive to variations in tobacco smoke exposure seems warranted. In this respect, it is noteworthy that lymphocytes also do not seem to be very sensitive to other types of genetic damage. DNA adducts or HPRT mutant frequency are also only moderately elevated in heavy smokers.[29,30]

Micronuclei in sputum[31] or bronchial brushings[32] have been reported to be 3-fold higher in smokers than non-smokers. Micronuclei in exfoliated epithelial cells reflect the extent of chromosome breakage due to mutagenic exposure, when the cells were dividing a few days

or weeks earlier, in the basal layer of the epithelium of the tracheobronchial tree.[33] The mean micronuclei counts in our study in healthy volunteers are lower than in studies using hospitalized patients.[31,32] Benner et al.[34] also observed lower micronuclei counts in healthy smokers undergoing bronchoscopy than in patients. The previous studies[31,32] also did not describe their scoring criteria, and counts for 'high certainty micronuclei' may be much lower than counts including 'medium certainty micronuclei'.[35] Within our group of smokers, the micronuclei parameter is not associated with smoking intensity, as was reported previously using counts in only 500 sputum cells in patients.[31] The lack of sensitivity for micronuclei in sputum in our study may be attributed to a large random sampling site variation, since expectorated cells may originate from all locations in the tracheobronchial tree. Studies using buccal micronuclei[33,35] and bronchial brushings[34] have indeed demonstrated major sampling site variations.

Both micronuclei and SCEs reflect DNA damage, but the exact molecular mechanisms are not known. Micronuclei are considered to reflect chromosome breakage,[33] whereas SCEs are considered to be due to perturbations in the DNA that persist through DNA replication.[36] *In vitro* studies indicate that the mechanisms may be mediated by free radicals and oxidants but can also involve other pathways.[37-40] The absence of associations of the vitamins C and E or carotenoids with SCEs and micronuclei does not support an involvement of free radicals and oxidants *in vivo*. However, there are numerous other non-enzymatic and enzymatic antioxidants that we did not measure,[41] blood levels may not reflect long-term antioxidant levels in lymphocytes or lung tissue, and the range of antioxidant levels in our study may not have been sufficiently large to demonstrate associations. Also, large random variations both in micronuclei and SCE counts may introduce bias towards the null and thus obscure a possible weak association.

The results for the GST-μ phenotype indicate that a part of the variation in SCE counts in smokers is genetically determined, since the GST-μ isozyme is inherited in an autosomal dominant fashion.[42] Glutathione S-transferases detoxify reactive electrophiles, in particular epoxides,[43] and GST-μ deficiency may imply a more limited capacity for detoxification and more carcinogen-mediated DNA damage. Our results for the SCE measure support this hypothesis and suggest that increased DNA damage in GST-μ deficient heavy smokers may be involved the association between GST-μ and lung cancer that is observed in case-control studies,[8,44-46] as we have previously discussed in more detail.[12] One of the studies showed a clear inverse relation between GST-μ deficiency and lung cancer in heavy smokers but not in light smokers,[18] which seems to correspond with our data. Another study[46] was more equivocal but also observed an inverse relation (although not statistically significant) only in heavy smokers. Zhong[45] observed an inverse relation for squamous carcinoma but not for adenocarcinoma of the lung. The results for the micronuclei counts do not support this hypothesis since micronuclei counts were even somewhat lower in GST-μ-deficient subjects. The different results for the GST-μ analysis suggest that micronuclei and SCE may be different biological phenomena; although epoxides may contribute to *in vivo* SCE induction, this may not be the case for micronuclei.

The concept that SCEs and micronuclei reflect different biological phenomena is also supported by the lack of an association between micronuclei and SCEs, as depicted in figure 3. Both micronuclei and SCEs are sensitive to carcinogens in experimental *in vivo* and *in vitro* models,[36,47,48] but there is little information on correlations between SCEs and micronuclei in these models to compare with our epidemiological observations. Here again, caution in interpretation of our data is required since unexplained random variation in both parameters may have obscured a weak association. Also, differences in tissues and time frame that the two cytogenetic parameters reflect may have contributed to the absence of a relation.

This study has evaluated the application of a number of biomarkers in a cross-sectional study in smokers and non-smokers. SCEs and micronuclei have been successfully used in previous studies to demonstrate differences in DNA damage between smokers and non-smokers. For the SCE measure, our study confirms these previous studies. In addition, the SCE measure in our study could be used to demonstrate differences in cytogenetic damage between smokers with or without a genetically determined detoxification enzyme. Our results do, however, demonstrate that both SCE and micronuclei have only limited or no sensitivity to variations in cigarette smoke exposure within a group of smokers. This limited sensitivity may be partly attributed to large variations of as yet unknown origin that we observe between persons in both SCE and micronuclei. This presumably random variation makes it difficult to unambiguously interpret the absence of relations (e.g. between antioxidants and cytogenetic damage) that we observe in this study. More information on biological, laboratory, and design factors that determine variations is necessary to be conclusive about the absence of associations.

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Increased cytogenetic damage in smokers deficient in glutathione S-transferase isozyme μ

Geert van Poppel, Nico de Vogel, Peter J. van Bladeren, Frans J. Kok

Abstract

Reduced expression of the μ-isozyme of glutathione S-transferase (GST; EC 2.5.1.18) has been associated with increased lung cancer risk. We studied the association between GST-μ expression and DNA damage as measured by sister chromatid exchanges (SCE) in healthy male smokers. SCE levels were higher in the 71 GST-μ-deficient smokers compared to the 83 non-deficient smokers (5.24 vs. 4.97 SCE per lymphocyte; p = 0.09). In smokers having high plasma cotinine levels (> median of 315 ng/ml), this μ-related difference was more pronounced (5.50 vs. 4.97; p = 0.01), whereas it was absent in smokers having low cotinine levels (4.95 vs. 4.97; p = 0.92). Increased cytogenetic damage in GST-μ deficient heavy smokers may thus explain the association between GST-μ expression and lung cancer.

Introduction

Although cigarette smoking causes almost all cases of bronchial carcinoma, many smokers do not develop lung cancer.[1] Individual susceptibility to lung cancer may be influenced by the enzymes that detoxify carcinogens in cigarette smoke.[1] Glutathione S-transferases (GST; EC 2.5.1.18) detoxify reactive electrophiles and may thus inhibit binding of carcinogens to DNA.[2] The three main types of glutathione S-transferase in humans are classes of isozymes designated α, μ and π.[2] The GST-μ isozyme is inherited in an autosomal dominant fashion,[3] and in about 50% of the human population, GST-μ activity is virtually absent.[3] There is evidence that expression of GST-μ is reduced in lung cancer patients.[4,5] This reduction in enzyme activity may be a result of the disease process, but it is hypothesized that increased smoking-induced cytogenetic damage in GST-μ deficient subjects explains the association between GST-μ deficiency and lung cancer.[6] To evaluate this hypothesis, we studied the association between GST-μ expression and cytogenetic damage in healthy males. Sister Chromatid Exchanges (SCE) in peripheral blood lymphocytes were measured as an endpoint of cytogenetic damage.

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Subject and methods

We studied healthy male volunteers, employed at the AMEV Insurance Company, the Taxation Office and the Power Company at Utrecht. The study was approved by an external Medical Ethical Committy and all participants gave their informed consent. Blood samples were taken by venapuncture into sterile heparinized Vacutainer tubes, and immediately stored at 4°C. Smokers (n = 155) reported consumption of > 15 cigarettes/day over > 2 years, whereas non-smokers (n = 66) reported never to have smoked. Plasma cotinine levels were determined by gas chromatography.[7] As cotinine is a good marker of smoking behaviour,[1] smokers were divided into either 'heavy' or 'light' smokers at the median of plasma cotinine levels. Presence or absence of GST-μ was established in heparinized whole blood using an enzyme-linked immunosorbent assay (MUKIT, Medlabs, Dublin, Ireland). One smoker was excluded from the analyses because the GST assay was ambiguous. None of the subjects reported exposure to chemicals or medications that could influence SCE levels.

Blood cultures for determination of Sister Chromatid Exchange in lymphocytes[8] were set up within 2-6 hours after venapunction, after the blood was allowed to reach room temperature for 30 minutes Heparinized whole blood (0.5 ml) was added to 4.4 ml prewarmed RPMI 1640 medium (Flow) containing 20% fetal calf serum (inactivated for 30 minutes at 56°C), 2.5% phytohaemagglutinin (HA-15 Welcome), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. 5-Bromo-2-deoxyuridine was added to a final concentration of 10 μg/ml. The blood was cultured in the dark at 37°C in T-25 culture flasks (Costar) in 5% CO_2 for 68 ± 1 hour. Colcemid was added at a final concentration of 0.2 μg/ml for the last 2 hours of incubation. The cells were collected by centrifugation, treated with hypotonic KCl (0.075 M) for 8 minutes to spread the chromosomes and to haemolyse the red blood cells, and fixed three times with methanol/acetic acid (3:1). After overnight storage in the dark at 4°C, cells were transferred to microscopic slides and air-dried. Preparations aged for 3 days and were stained by the fluorescence plus Giemsa technique[8] to obtain harlequin chromosomes. For each subject SCE in 50 second-division metaphases were scored as colour changes in the longitudinal direction of the chromatid, excluding the centromere. Only nuclei with 46 chromosomes were scored. Individual data are the mean counts of 50 metaphases. Differences between GST-μ-deficient and non-deficient subjects were evaluated using a two-tailed unpaired t-test and multivariate regression analysis.

Results and discussion

As we expected, smokers had a higher number of SCE per lymphocyte than non-smokers (5.08 vs. 4.66, $p < 0.001$, see also figure 1). An approximately 10% increase in SCE levels in smokers is in accordance with some, but not all studies.[1] In 24 out of 27 studies, SCE were increased in smokers, levels being 10-88% higher that in non-smokers.[1] It should be

noted that SCE levels observed in several studies cannot readily be compared, as laboratory protocols are not standardized.[8] The non-smokers were younger than the smokers and had a lower body mass index. However, multivariate regression analysis did not reveal significant associations between age or body mass index and SCE levels, and the significant difference in SCE level between smokers and non-smokers persisted after controlling for age and body mass index.

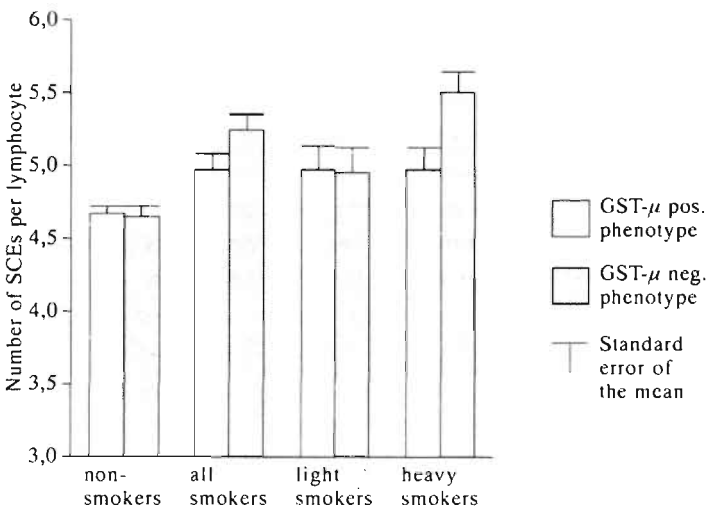

Figure 1. Levels of SCE in cultured lymphocytes of GST-μ-positive and -negative smokers and non-smokers. Characteristics of non-smokers, smokers, light smokers and heavy smokers have been given in the text.

Forty-five per cent of all subjects were deficient in GST-μ, which agrees well with previous estimates.[3] Age, body mass index, cigarette consumption and cotinine levels were almost similar for μ-positive or μ-negative subjects (table 1 and table 2). In the 'heavy' smokers, reported cigarette consumption was only 14% higher (19.8 vs. 22.5, $p < 0.01$) than in the 'light' smokers, indicating that self-reported cigarette consumption yields only a rough estimate of actual exposure. Figure 1 shows levels of SCE in the smoking and non-smoking groups. In non-smokers no differences between GST-μ phenotypes were observed (4.67 vs. 4.65, $p = 0.79$). In the smokers, however, the GST-μ negative phenotype was associated with higher SCE levels (5.24 vs. 4.97, $p = 0.09$). This μ-related difference in SCE was more pronounced in heavy smokers (5.50 vs. 4.97, $p = 0.01$), whereas it was absent in light smokers (4.95 vs. 4.97, $p = 0.92$). In the heavy smokers, the μ-related difference in SCE was similar in multiple regression analysis adjusting for age, body mass index, duration and quantity of smoking, and cotinine levels (5.47 vs. 4.99, $p = 0.02$). In the light smokers, the multivariate adjustment also yielded similar results (4.94 vs. 4.98, $p = 0.87$).

Table 1. Characteristics of GST-μ-positive and -negative smokers and non-smokers (mean ± SD).

	GST-μ-positive non-smokers (n = 39)	GST-μ-negative non-smokers (n = 27)	GST-μ-positive smokers (n = 81)	GST-μ-negative smokers (n = 73)
Age (years)	33.5 ± 8.5	37.2 ± 10.8	39.5 ± 9.0	38.6 ± 10.5
Body Mass Index (kg/m^2)	23.7 ± 2.3	24.1 ± 2.6	24.4 ± 3.2	24.7 ± 2.9
Plasma Cotinine (μg/ml)	1.0 ± 1.0	0.9 ± 0.6	323.1 ± 120.0	334.9 ± 114.8
Cigarettes/day	0	0	20.8 ± 5.7	21.5 ± 7.3
Packyears[1]	0	0	22.3 ± 12.1	22.3 ± 12.7

[1] Reported number of packs smoked per day x number of years smoked.

Table 2. Characteristics of GST-μ-positive and -negative light smokers (plasma cotinine < 315 μg/ml) and heavy smokers (plasma cotinine > 315 μg/ml) (mean ± SD).

	GST-μ positive light smoker (n = 41)	GST-μ negative light smoker (n = 35)	GST-μ positive heavy smoker (n = 40)	GST-μ negative heavy smoker (n = 38)
Age (years)	38.5 ± 9.5	37.2 ± 10.8	40.5 ± 8.4	39.5 ± 10.7
Body Mass Index (kg/m^2)	25.0 ± 3.6	24.8 ± 2.6	23.7 ± 2.7	24.7 ± 3.1
Plasma Cotinine (μg/ml)	230.6 ± 64.4	236.2 ± 58.3	415.9 ± 86.4	425.9 ± 69.4
Cigarettes/day	20.2 ± 4.8	19.0 ± 4.7	21.4 ± 6.5	23.7 ± 8.5
Packyears[1]	20.8 ± 12.2	18.3 ± 9.9	23.9 ± 11.9	25.9 ± 14.0

[1] Reported number of packs smoked per day x number of years smoked.

The 10% increase in SCE levels, that we observed in GST-μ-deficient heavy smokers, is of the same order of magnitude as the difference we observed between smokers and non-smokers. These results thus show for the first time that a genetic deficiency in detoxication of xenobiotics is associated with substantially increased cytogenetic damage in a human population exposed to carcinogens. Biotransformation of xenobiotics involves both the oxidative phase I metabolism, usually catalysed by cytochrome P450 isoenzymes, and phase II conjugation reactions. Reactive intermediates are often produced through phase I metabolism and subsequently detoxicated by phase II enzymes.[9] The phase II glutathione S-transferases in particular detoxify epoxides by conjugating them to glutathione.[2,10] Cigarette smoke is a complex mixture of many compounds.[1] Substrates for GST-μ derived from cigarette smoke constituents include styrene oxide[10] and the mutagenic benzo[a]pyrene (BP) metabolites BP 4,5-oxide,[11] and anti-BP-7, 8-diol-9, 10-oxide,[11] of which the latter has been shown to activate C-Ha-ras-1 protooncogene *in vitro*.[12] Also, GST-μ shows a particularly high specificity for *trans*-stilbene oxide,[2] which, however, is

not known to occur in cigarette smoke. Nevertheless, a recent *in vitro* study showed SCE induction by *trans*-stilbene oxide to be inversely associated with GST-μ,[6] which is in accordance with our findings.

DNA damage is considered crucial in carcinogenesis,[13] and SCE are highly sensitive to the mutagenic activity of a great number of carcinogens.[14] Numerous studies have shown that SCE in lymphocytes are increased in smokers as compared to non-smokers.[1,15] Also, SCE levels were reported to be increased in lung cancer patients as compared with controls matched for smoking habits.[15] These observations are suggestive, but there have not been any follow-up studies to provide direct evidence for a predictive value of SCE in the development of lung cancer. Moreover, the GST-μ assay in this study was performed in peripheral blood. GST-μ deficiency in peripheral blood cells is assumed to correspond with GST-μ deficiency in other tissues. For human liver tissue, this has indeed been shown,[16] but similar data on lung tissue are lacking. Our results do, however, show a striking similarity with a study in lung cancer patients,[4] which reported GST-μ deficiency (assessed in peripheral blood) to be associated with lung cancer in heavy smokers, but not in light smokers. This seeming relevance of GST-μ only at high exposures may be explained by other forms of GST isozymes. This may be exemplified by the fact that both μ and π class isozymes are efficient at conjugating benzo[a]pyrene diol epoxide, whereas the α class transferase shows only weak activity. The μ and π isozymes display comparable K_M and V_{max} values with (±)-anti-BDPE as substrate.[11] For the isozyme π no deficiencies have been described and the organism is thus, even in the absence of the μ isozyme, able to detoxify the epoxide, but with a more limited capacity.[17]

We conclude that increased DNA damage in GST-μ-deficient heavy smokers may be the aetiological explanation of the epidemiological association between GST-μ phenotype and lung cancer. Clearly, GST-μ phenotype cannot explain all of the variation in cytogenetic damage and lung cancer risk among smokers. Interestingly, two genetically controlled phase I enzymes, aryl hydrocarbon hydroxylase (cytochrome P450IA1) and debrisoquine hydroxylase (cytochrome P450IID6), have also been reported to be correlated with susceptibility to lung cancer in smokers, though the results have not been unambiguous.[18,19] The genetically determined balance between phase I and phase II enzymes may therefore be important with regard to susceptibility to lung cancer and deserves further study.

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No influence of beta-carotene on smoking induced DNA damage as reflected by sister chromatid exchanges

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Abstract

The putative cancer preventive potential of β-carotene may be explained by its antioxidant capacity to prevent free radical-induced DNA damage. To evaluate this hypothesis, we studied the effect of 14-weeks of β-carotene supplementation on the frequency of sister chromatid exchanges (SCE) in lymphocytes in 143 heavy smokers in a randomized, double-blind, placebo-controlled intervention trial. Age, smoking habits and pre-treatment blood levels of cotinine, β-carotene, retinol, and vitamins C and E were similar in the placebo group (n = 73) and the treatment group (n = 70). Plasma β-carotene levels increased 13-fold in the treatment group during intervention, whereas the other parameters remained stable in both groups. Initial SCE levels were similar in the treatment and placebo groups (5.10 ± 0.98 vs. 5.00 ± 0.99 SCE per lymphocyte). During the intervention, both groups showed an almost identical decrease, and at the end of the intervention period there was no difference in SCE levels between the treatment and the placebo groups (4.37 ± 0.38 vs. 4.24 ± 0.37 SCE per lymphocyte).

This study shows no protective effect of β-carotene on DNA damage as reflected by sister chromatid exchanges in lymphocytes. Our results thus do not yield support for a cancer preventive mechanism of β-carotene involving this form of DNA damage. It cannot be excluded, however, that β-carotene prevents other forms of smoking induced DNA damage, affects other tissues, or is preventive in later stages of carcinogenesis.

Introduction

The adverse effects of smoking on the development of lung cancer have been well documented.[1] However, a majority of smokers do not develop lung cancer. Smokers may be partially protected by components in the diet, and several studies suggest that β-carotene might reduce the risk of lung cancer.[2]

β-carotene, an antioxidant, is hypothesized to be effective through its ability to quench free radicals.[3,4] Components of cigarette smoke may induce (*in vivo*) formation of free radicals which may damage DNA.[5] DNA damage is regarded as a crucial step in chemical carcinogenesis.[6] Increased cytogenetic damage *in vivo*, as measured by sister

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chromatid exchanges (SCEs) in lymphocytes, may thus reflect increased cancer risk. Thus, SCE levels have been reported to be increased in smokers and in lung cancer patients.[1,7]

To test the hypothesis that β-carotene protects against smoking induced DNA damage, we performed a randomized double-blind placebo-controlled intervention trial. We evaluated the effect of β-carotene suppletion on DNA damage in smokers measured by the sister chromatide exchange measure.

Subjects and methods

Study design

Healthy male employees of the AMEV Insurance Company, the Taxation Office and the Power Company, all based at Utrecht were asked to volunteer for the intervention trial. All participants had smoked at least 15 cigarettes/day for over 2 years, did not use vitamin preparations containing retinol or carotenoids, or medications known to influence SCE levels. Moreover, they reported that they were not exposed to chemicals during working or leisure time. The volunteers were prestratified on age, duration and quantity of smoking and randomly assigned to either β-carotene (20 mg capsules, Hoffmann-La Roche, Mijdrecht, The Netherlands) or placebo treatment.

Blood samples were collected before and after the 14-weeks treatment. The participants were instructed to take capsules daily with dinner; 2 capsules/day during the first 2-weeks, followed by 1 capsule/day for the next 12-weeks. Every 4-weeks, the participants were sent their next strip of 28 capsules, and were asked to return the used strips with the remaining capsules to monitor compliance. In addition, β-carotene was determined in a blood sample taken after 7-weeks of treatment.

Initially, 163 smokers volunteered to participate; 83 were assigned to the placebo treatment, 80 to the β-carotene treatment. During the trial, 13 smokers (6 placebo, 7 β-carotene) terminated participation because of stopping smoking (n = 4), illness or accident (n = 3), private circumstances (n = 1), forgetting to take capsules (n = 2), or without giving a reason (n = 3). Of the 150 smokers who completed the trial, another 7 (4 placebo, 3 β-carotene) dropped out because microscopic preparations were inadequate, leaving 143 subjects (73 placebo, 70 β-carotene) for data analysis.

Sister chromatid exchanges

Blood cultures for determination of SCEs in lymphocytes[8] were set up within 2 to 6 hours after venipuncture, after the blood had reached room temperature for 30 minutes. Heparinized whole blood (0.5 ml) was added to 4.4 ml prewarmed RPMI 1640 medium (Flow, Irvine, UK) containing 20% FCS (inactivated for 30 minutes at 56°C), 2.5% phytohaemagglutinin (HA-15 Welcome, Weesp, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. 5-bromo, 2-deoxyuridine was added to a final concentration of 10 μg/ml. The blood was cultured in the dark at 37°C in T-25 culture flasks (Costar, Cambridge, MA) in 5% CO_2 for 68 ± 1 hour. Colcemid was added to a final

concentration of 0.2 μg/ml for the last 2 hours of incubation. The cells were collected by centrifugation, treated with hypotonic KCl (0.075 M) for 8 minutes to spread the chromosomes and to heamolyse the RBC, and fixed 3 times with methanol-acetic acid (3:1). After overnight storage in the dark at 4°C, cells were transferred to microscopic slides and air-dried. Preparations aged for 3 days and were stained by the fluorescence plus Giemsa technique[8] to obtain harlequin chromosomes. For each subject, SCEs in 50 second-division metaphases were scored as colour changes in the longitudinal direction of the chromatid, excluding the centromere. Only nuclei with 46 chromosomes were scored. Individual data are the mean counts of 50 metaphases.

Blood parameters

Directly after venipuncture, blood samples were stored in the dark at 0 to 4°C. Lymphocyte counts were obtained in Na-EDTA blood samples, after 3 to 5 hours storage, with the use of an Sysmex K-1000 Haematology Analyzer (Toa, Tokyo, Japan). After 20 to 23 hours overnight dark storage at 4°C, a separate evacuated tube was opened to determine the sum of ascorbic acid + dehydro-ascorbic acid (vitamin C) in whole blood by HPLC with fluorometric detection.[9] All-*trans* retinol, α-tocopherol, retinol, β-carotene and total carotenoids were assayed in EDTA plasma (stored at −80°C) by HPLC with colorimetric detection.[10] Plasma cotinine levels were determined by gas chromatography.[11]

Data analysis

Initial values for all variables and changes in these variables during the intervention period were compared between the β-carotene and placebo groups using the unpaired Student's t-test. Associations between variables were evaluated by univariate linear regression analysis: correlation coefficients are Pearson coefficients. Multiple regression analysis was used to correct for residual confounding in the comparison between the β-carotene and placebo groups. Data analysis was performed with the use of the BMDP statistical software package.[12]

Results

The placebo and β-carotene groups had comparable age (39.1 ± 10.1 vs. 39.2 ± 9.5 years) and smoking habits (20.8 ± 6.9 vs. 21.4 ± 5.8 cigarettes/day). Table 1 shows that the placebo and β-carotene groups are comparable for all other characteristics and that, except for plasma β-carotene, only minor changes occurred during the intervention. In accordance with the stable cotinine levels, only one smoker (placebo group) reported having changed his smoking habits during the trial. His plasma cotinine levels, however, hardly changed (349 vs. 313 μmol/l respectively). The mean body mass index was similar in the placebo group (24.5 ± 3.0 kg/m²) and the β-carotene group (24.6 ± 3.1 kg/m²), and all participants but one reported stable weights during the trial (one placebo participant: BMI from 24.9 to 22.2 kg/m²). After 7-weeks, the mean plasma β-carotene level had increased 13-fold to

4.44 ± 2.12 μmol/l in the supplemented group (0.28 ± 0.18 in the placebo group), and remained stable until the end of the trial (table 1). Plasma β-carotene levels increased in all supplemented subjects but one. For this subject, pill counts to evaluate compliance were not available. For all other supplemented volunteers, the minimum increase in plasma β-carotene after supplementation was 1.7-fold (from 0.44 to 0.74 μmol/l), and all but 5 supplemented subjects had after-treatment plasma levels above 1.0 μmol/l. Twenty participants (19 β-carotene and, surprisingly, one placebo) reported having observed skin yellowing during the trial. Pill counts showed that 91% of all capsules were taken on average (data for 129 subjects); all but 10 participants took more than 75% of their capsules.

Table 1. Initial and final values (mean ± s.d.) of blood parameters during a 14-weeks intervention trial in male smokers, assigned to either β-carotene or placebo treatment.

	Placebo group (n = 73)		β-carotene group (n = 70)	
	initial values	final values	initial values	final values
Lymphocyte count (10^9cells/l)[1]	2.29 ± 0.63	2.21 ± 0.56	2.24 ± 0.58	2.15 ± 0.48
Blood vitamin C (μmol/l)[2]	35.2 ± 17.6	36.1 ± 17.9	37.3 ± 17.2	35.2 ± 16.2
Plasma retinol (μmol/l)	2.31 ± 0.60	2.25 ± 0.46	2.33 ± 0.48	2.36 ± 0.57
Plasma α-tocopherol (μmol/l)	30.2 ± 6.6	31.7 ± 6.8	31.4 ± 6.0	31.8 ± 6.1
Plasma β-carotene (μmol/l)	0.30 ± 0.20	0.28 ± 0.19	0.33 ± 0.16	4.36 ± 2.32*
Plasma total carotenoids (μmol/l)	1.57 ± 0.57	1.63 ± 0.62	1.59 ± 0.62	5.36 ± 2.44*
Plasma cotinine (μg/l)	318.7 ± 126.4	309.0 ± 130.6	335.6 ± 106.9	322.8 ± 105.3

* β-carotene group significantly different from placebo group, $p < 0.0001$.
- [1] nine missing values. - [2] five missing values.

In the baseline measurement, significant correlations with SCE levels were observed for plasma cotinine ($R = 0.19$, $p = 0.02$), cigarette consumption ($R = 0.25$, $p = 0.002$) and age ($R = 0.15$, $p = 0.06$). No other baseline variables in table 1 showed associations with SCE levels ($R < 0.08$, $p > 0.3$ for all of these). The changes in variables given in table 1 were not associated with changes in SCE levels during the intervention ($R < 0.07$, $p > 0.4$ for all variables). No association was detected between the individual SCE levels before and after the intervention trial ($R = 0.05$, $p = 0.6$), which indicates a very large personal variation during the trial.

At baseline, the SCE levels in the β-carotene group and the placebo group were similar (table 2). During the intervention, these levels showed an almost identical decrease in both groups, and the SCE levels after treatment were also similar in both groups. This comparison yielded the same results when supplemented subjects with after-treatment β-carotene levels below the median (4.11 μmol/l) were excluded (initial SCE count per lymphocyte 5.16 ± 1.0; final SCE level 4.38 ± 0.4). Adjustment for age, cotinine level,

lymphocyte count, and blood level of vitamins A, C and E did not influence the comparability in SCE levels between both groups before or after the intervention.

Table 2. Frequency of sister chromatid exchanges (mean ± s.d. per lymphocyte) and changes in these frequencies during a 14-weeks intervention trial in male smokers, assigned to either β-carotene or placebo treatment.

	Placebo group (n = 73)	β-carotene group (n = 70)
SCEs before intervention	5.00 ± 0.99	5.10 ± 0.98
SCEs after intervention	4.24 ± 0.37	4.37 ± 0.38
Change in SCEs (after-before)	−0.76 ± 1.04	−0.74 ± 1.02

Discussion

This trial in heavy smokers shows no effect of β-carotene supplementation on the level of sister chromatid exchanges in lymphocytes. This indicates that β-carotene does not influence the DNA lesions reflected by sister chromatid exchanges in circulating lymphocytes.

The lack of an effect of β-carotene in this study can hardly be attributed to methodological shortcomings, since randomization was successful, withdrawals were limited, and compliance, reflected both by pill counts and by blood parameters, was very good. Moreover, the β-carotene and placebo groups were always equally represented on measurement days, to minimize bias from between-run variations. It also seems unlikely that the dose of β-carotene was too low as 20 mg/day is equivalent to 5 to 10 times the normal intake, and plasma levels in the β-carotene group increased dramatically. Moreover, we observed no tendency towards a favourable effect, even after exclusion of supplemented subjects with plasma β-carotene values below the median after treatment. Based on our results, there is only 5% chance of a more than 0.1% lower SCE value after treatment in the β-carotene group, as compared with the placebo group. For comparisons: we observed 10% lower SCE levels in 66 non-smokers as compared with the smokers during the baseline study.[13] The SCE levels observed are in accordance with some studies, whereas other studies report higher levels in smokers.[1] SCE levels observed in different studies, however, cannot readily be compared, as laboratory protocols are not standardized.[8]

The absence of an effect of β-carotene also can not be explained by the treatment time being insufficient to influence persistent lesions. If the lesions giving rise to SCEs did indeed persist unrepaired, then SCE levels in people would remain fairly constant over time. In contrast, we observed a decline in SCEs both in the placebo and the β-carotene groups, as well as large personal variations. Information on temporal fluctuations in SCE

levels is scarce,[14] but the decline in SCE levels may be explained by seasonal variations. Although we expended much effort on standardizing laboratory protocols, inevitable shifts in culturing conditions (e.g., ageing of the frozen batch of FCS), may also have contributed to the observed decline. The somewhat unexpected decline does not, however, influence the interpretation of the effect of β-carotene, as the trial was fully placebo-controlled, and both groups were always equally represented in all culturing, staining and scoring runs. By analogy, the large personal variations cannot have influenced the interpretation of β-carotene effects at a group level in this randomized, placebo-controlled trial. As we were not able to standardize the time of blood sampling in this epidemiological study setting, circadian rhythms in SCE levels[14] may explain the large personal variations. Here again, placebo and β-carotene participants were randomly divided over sampling times during each sampling day.

It is tempting to interpret the lack of effect of β-carotene in this study as evidence for the absence of a cancer preventive potential of this provitamin. However, caution is called for when interpreting our data. In mouse mammary cell culture, β-carotene has been shown to reduce SCEs induced by chemical carcinogens.[15] *In vitro* studies have shown that SCEs are sensitive to the mutagenic activity of a great number of carcinogens.[16] The precise molecular mechanism for *in vivo* SCE induction by inhaled cigarette smoke, however, has never been elucidated.[13] This *in vivo* induction may be primarily caused by pathways not involving free radicals or oxidation reactions. This possibility is supported by *in vitro* experiments on SCE induction by cigarette smoke.[17] If so, this would explain the absence of an effect of β-carotene without precluding the protective potential in other DNA damaging pathways. Indeed, we recently reported evidence that hydrocarbon epoxides may be important in *in vivo* SCE induction by cigarette smoke,[13] whereas such epoxides are not believed to be formed or to damage DNA through mechanisms involving free radicals.[18,19,20]

Though SCEs have been consistently reported to be higher in smokers than in non-smokers,[1] and even have been found to be elevated in lung cancer patients,[7] the predictive value of SCEs in lung cancer development remains to be demonstrated. SCEs clearly reflect potentially deleterious effects of cigarette smoke, but the relevance of this measurement in lymphocytes to biological effects in lung tissue remains uncertain. This site specificity may be especially important with regard to β-carotene, as the observational evidence for the protective effect of this compound is remarkably consistent for lung cancer, and far more equivocal for other sites.[2] The site specificity may be explained if β-carotene is primarily effective in cells directly exposed to tobacco components. Indeed, Stich et al.[21] have shown that β-carotene reduces cytogenetic damage (micronuclei) in oral mucosal cells in tobacco chewers.

Our data in human volunteers exposed to cigarette smoke do not correspond with laboratory studies which indicate a role for β-carotene in antimutagenesis and prevention of malignant transformation.[4] However, there are also indications that β-carotene may affect later stages of carcinogenesis.[22] Studies in humans using other, more specific, biomarkers

of DNA damage may further elucidate the role of β-carotene in DNA damage and early-stage carcinogenesis. In this respect, recent developments in the measurement of DNA adducts at a molecular level[23] may offer exciting opportunities for future epidemiological research.

We conclude that our results using the SCE measurements in lymphocytes do not yield support for the cancer preventive potential of β-carotene. Our study shows that supplementary β-carotene is not able to prevent all forms of smoking-induced DNA damage. It cannot be excluded however, that β-carotene prevents other forms of smoking induced DNA damage, affects other tissues, or is preventive in later stages of carcinogenesis.

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Appendix to chapter 5: Analysis stratified for GST-μ.

In chapters 3 and 4, an association between glutathione S-transferase phenotype and sister chromatid exchanges in lymphocytes was demonstrated. The table therefore shows the results for the SCE measure before and after β-carotene intervention in the GST-μ negative and GST-μ positive groups. In both groups, there is no effect of β-carotene on sister chromatid exchanges.

	GST-μ negative		GST-μ positive	
	Placebo	β-carotene	Placebo	β-carotene
	n = 33	n = 37	n = 39	n = 33
SCE's before intervention	5.08 ± 0.92	5.32 ± 0.98	4.96 ± 1.04	4.86 ± 0.95
SCE's after intervention	4.34 ± 0.37	4.42 ± 0.33	4.17 ± 0.37	4.30 ± 0.43
Changes in SCE's	−0.74 ± 1.00	−0.90 ± 1.05	−0.80 ± 1.09	−0.55 ± 0.98

Beta-carotene supplementation in smokers reduces the frequency of micronuclei in sputum

Geert van Poppel, Frans J. Kok, Ruud J.J. Hermus

Abstract

β-carotene has been hypothesised to reduce lung cancer risk. We studied the effect of 14-weeks of β-carotene supplementation (20 mg/day) on the frequency of micronuclei in sputum in 114 heavy smokers in a double-blind trial. Micronuclei reflect DNA damage in exfoliated cells and may thus provide a marker of early-stage carcinogenesis.

Pre-treatment blood levels of cotinine, β-carotene, retinol and vitamins C and E were similar in the placebo group (n = 61) and the treatment group (n = 53). Plasma β-carotene levels increased 13-fold in the treatment group during intervention. Initial micronuclei counts (per 3000 cells) were higher in the treatment group than in the placebo group (5.0 vs. 4.0, $p < 0.05$). During intervention, the treatment group showed a 47% decrease, whereas the placebo group showed a non-significant decrease (16%). After adjustment for the initial levels, the treatment group had 27% lower micronuclei counts than the placebo group at the end of the trial (95% CI: 9-41%).

These results indicate that β-carotene may reduce lung cancer risk in man by preventing DNA damage in early-stage carcinogenesis.

Introduction

The scientific interest in the role of carotenoids and retinoids in the prevention of human cancer has culminated in recent years.[1,2] Especially for lung cancer, epidemiological studies have consistently shown inverse associations between plasma or dietary β-carotene and cancer incidence.[3,4,5] Since these studies cannot prove causal associations. two large randomized trials are currently conducted to evaluate the beneficial effect of β-carotene on human cancer development.[6,7] These intervention studies, however, provide little information on biological mechanisms.

Damage to DNA is considered a crucial mechanism in cancer development.[8] Micronuclei, DNA fragments in exfoliated cells, may thus provide a marker of early-stage carcinogenesis in target tissues.[9,10] In cigarette smokers, elevated micronuclei counts in expectorated sputum[11] or bronchial brushings[9] are thought to reflect increased lung cancer risk.

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So far, no studies have investigated the effect of β-carotene supplementation on sputum micronuclei, as a reflection of lung cancer risk. β-carotene has been shown to reduce micronucleated buccal mucosal cells in tobacco chewers[10,12,13] and may thus reduce risk for oral cancer. These trials, however, did not measure plasma levels of β-carotene and retinol. Moreover, other antioxidant vitamins may modify the effects of β-carotene.[2] We now report on a 14-weeks, double-blind, randomized placebo-controlled trial of the effect of β-carotene on sputum micronuclei in 114 heavy smokers. We measured plasma cotinine as a marker for tobacco exposure and monitored blood levels of β-carotene, retinol and the antioxidant vitamins C and E.

Subjects and methods

Study design

Healthy male employees of the AMEV Insurance Company, the Taxation Office and the Power Company at Utrecht, Netherlands, were asked to volunteer for the intervention trial, which was approved by an External Review Board for experiments with human volunteers. All participants had smoked at least 15 cigarettes/day for over 2 years, did not use preparations containing retinol or carotenoids, and did not report exposure to chemicals during working or leisure time. The volunteers were prestratified by age, duration and quantity of smoking and randomly assigned to either β-carotene (20 mg capsules, F. Hoffmann-La Roche) or placebo treatment.

Blood and sputum samples were collected before and after the 14-weeks treatment. The participants were instructed to take capsules daily with the evening meal, 2 capsules/day during the first 2-weeks, followed by one capsule/day over the next 12-weeks. Every 4-weeks, the participants were sent their next strip of 28 capsules, and were asked to return the used strips with the capsules not taken to monitor compliance. In addition, β-carotene was determined in a blood sample taken after 7-weeks of treatment.

Initially, 163 smokers volunteered to participate; 83 were assigned to placebo treatment, 80 to β-carotene treatment. During the trial, a total of 13 smokers (6 placebo, 7 β-carotene) discontinued participation because of stopping smoking (n = 4), illness or an accident (n = 3), personal circumstances (n = 1), forgetting to take capsules (n = 2), or without giving a reason (n = 3). Of the 150 smokers who completed the trial, 29 (13 placebo, 16 β-carotene) failed to produce sputum samples. In addition, insufficient cells could be evaluated in 7 subjects (3 placebo, 4 β-carotene), leaving 114 subjects (61 placebo, 53 β-carotene) for data analysis.

Micronuclei in sputum

Sputum was collected and processed as described in detail by Saccomano et al. (1978).[14] Each participant received a careful individual instruction on how to produce a specimen from 'deep in the lungs'. Sputum was collected at home on three consecutive mornings, directly after rising and after carefully rinsing the mouth. The three, or minimally two

samples collected in preservative (50 ml 50% ethanol with 2% polyethylene glycol (Carbowax 1540, Merck)) were mixed, homogenized, centrifuged and smeared onto slides. The slides were stained with Feulgen and fast green, which is specific for DNA and strongly highlights micronuclei.[15]

For each subject, 3000 cells were examined and evaluated on the basis of the following criteria: shape and size typical of epithelial cells, a well defined nucleus and a clearly defined cytoplasm. The criteria in defining a micronucleus were: chromatin structure and colour intensity similar to those of the main nucleus; on focusing, the micronucleus must be on the same level as the nucleus, must be roundish and clearly included in the cytoplasm. The dimensions should be less than 1/5 of that of the main nucleus, and it should not be connected to it. Slides were screened at 400x magnification and micro-nucleated cells were examined at 1000x magnification. Slides were read coded/blinded by a single observer. Repeated blinded scoring of 9 samples yielded a good correlation (Pearson R = 0.86), with 2 of the 9 samples showing a difference of more than 1 micronucleus upon rescoring.

Blood parameters

Directly after venapuncture, non-fasting blood samples containing NaEDTA as anti-coagulant were stored overnight in the dark at 4°C for 20-23 hours. Directly after opening the evacuated tubes, the sum of L-ascorbic acid + dehydro-L-ascorbic acid (vitamin C) was assessed in wholeblood by HPLC with fluorometric detection.[16] All-*trans* retinol, α-tocopherol, β-carotene and total carotenoids were assayed in plasma (stored at −80°C) by HPLC with colorimetric detection.[17] Plasma cotinine levels were determined by gas chromatography.[18]

Data analysis

Initial baseline values and changes in these values during the intervention period were compared between the placebo group and the β-carotene group using the unpaired Student's t-test. Univariate log-linear Poisson regression was used to compare micronuclei counts between both groups, and to evaluate associations between micronuclei counts and other parameters. Percentual changes in micronuclei counts during the intervention trial were quantified by analyzing the final/initial micronuclei counts ratio in binomial regression. Multiple Poisson regression was used to quantify the difference in micronuclei between the placebo and β-carotene group after correction for incomplete randomization. All data analysis were performed using the BMDP and GENSTAT packages.[19,20]

Results

Table 1 shows that the placebo and β-carotene groups are comparable for all characteristics and that, except for plasma β-carotene, only minor changes occurred during the intervention trial. In accordance with the stable cotinine levels, only one smoker (placebo

group) reported to have changed his smoking habits during the trial. His plasma cotinine levels, however, hardly changed (349 and 313 nmol/ml respectively). The mean body mass index (BMI) was similar in the placebo group (24.3 ± 3.0 kg/m²) and the β-carotene group (24.7 ± 3.3 kg/m²), and all participants but one reported stable weights during the trial (in one placebo participant BMI decreased from 24.9 to 22.2 kg/m²). Reported alcohol consumption was also similar in the placebo (13.4 g/day) and the β-carotene

Table 1. Baseline characteristics (mean ± SD) and changes in these characteristics during a 14-weeks intervention trial in male smokers, assigned to either β-carotene or placebo treatment.

	Placebo group (n = 61)		β-carotene group (n = 53)	
	baseline values	change (after-before)	baseline values	change (after-before)
Age (yrs)	40.0 ± 10.1	n.a	40.2 ± 9.1	n.a
Number of cigarettes/day	20.8 ± 6.7	n.a	21.7 ± 6.4	n.a
Years of smoking	21.9 ± 10.5	n.a	22.0 ± 9.1	n.a
Blood vitamin C (μmol/l)	37.6 ± 18.8	−0.4 ± 16.7	38.2 ± 17.2	−1.1 ± 16.0
Plasma retinol (μmol/l)	2.33 ± 0.60	−0.06 ± 0.42	2.38 ± 0.55	−0.01 ± 0.41
Plasma α-tocopherol (μmol/l)	30.5 ± 7.1	1.4 ± 4.3	31.2 ± 7.0	0.5 ± 3.2
Plasma β-carotene (μmol/l)	0.28 ± 0.18	−0.02 ± 0.13	0.32 ± 0.16	3.79 ± 2.02*
Plasma total carotenoids (μmol/l)	1.56 ± 0.59	0.09 ± 0.43	1.49 ± 0.58	3.66 ± 1.93*
Plasma cotinine (ng/ml)	323.1 ± 122.6	−8.4 ± 72.8	332.8 ± 109.6	−8.0 ± 74.1

* β-carotene group significantly different from the placebo group, $p < 0.0001$.
n.a. = not applicable.

group (12.6 g/day). After 7-weeks, mean plasma β-carotene had increased 13-fold to 4.13 ± 1.79 μmol/l in the supplemented group (0.26 ± 0.15 μmol/l in the placebo group), and remained stable up to the end of the trial. The minimum increase in plasma β-carotene after supplementation was 1.7-fold (0.44 → 0.74 μmol/l), and all but 4 supplemented subjects had after treatment plasma levels above 1.0 μmol/l. Thirteen participants (12 β-carotene and, surprisingly, one placebo) reported to have observed skin yellowing during the trial, whereas one of the investigators, unaware of intervention status, noted 19 cases of skin yellowing (all β-carotene subjects). Pill counts showed that 92% of all capsules were taken (data for 103 subjects); all but 4 participants took more than 75% of their capsules. At baseline, the micronuclei counts were significantly higher in the β-carotene group than in the placebo group (figure 1 and table 2). After the intervention,however, the micronuclei counts were significantly lower in the β-carotene group (table 2). The β-carotene group thus showed a strong decrease in micronuclei counts, whereas the placebo group showed a minor, non-significant decrease (figure 2 and table 2). In the treatment group the decrease in micronuclei was similar (47%) in subjects with final β-carotene levels above and below the median of 4.1 μmol/l. To obtain an unbiased estimate of the intervention effect, we calculated the difference between the placebo group and the β-carotene groups after

intervention, allowing for the differences that existed between both groups before the intervention. After adjustment for initial micronuclei counts, the final micronuclei counts were estimated to be 27% lower in the β-carotene group than in the placebo group (95% CI: 9% - 41%). Adjustment for the baseline characteristics given in table 1 did not alter this estimate, since no associations were detected between micronuclei counts at baseline and any of the baseline characteristics listed in table 1, or alcohol consumption (All Pearson R < 0.14).

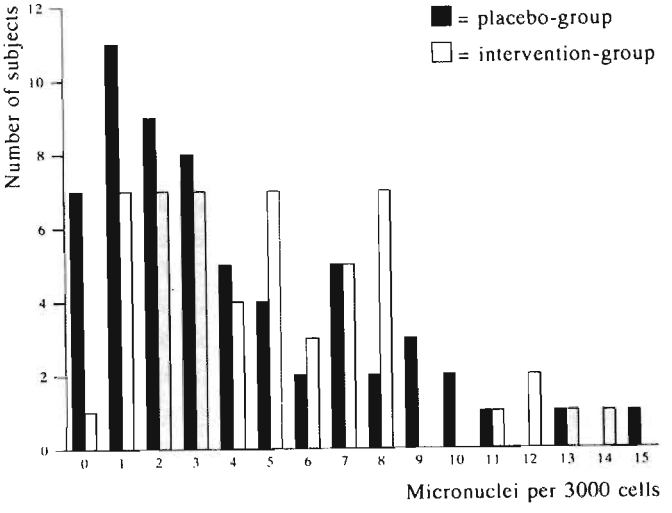

Figure 1. Distribution of micronuclei counts at baseline.

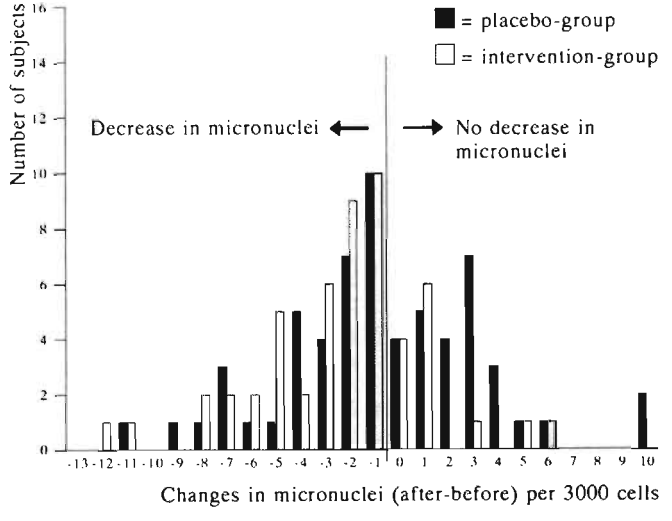

Figure 2. Changes in micronuclei counts.

Table 2. Micronuclei counts (mean per 3000 cells ± SD) and changes in micronuclei counts during a 14-weeks intervention trial in male smokers, assigned to either β-carotene or placebo treatment.

	Placebo group (n = 61)	β-carotene group (n = 53)
Micronuclei at baseline*	4.0 ± 3.5	5.0 ± 3.4
Micronuclei after 14-weeks treatment*	3.4 ± 3.3	2.6 ± 2.8
Change in micronuclei (after-before)*	−0.6 ± 4.0	−2.3 ± 3.4
% Change in micronuclei	−16	−47
(95% confidence interval)	(−31% - +1%)	(−57% - −35%

* β-carotene group significantly different from placebo group; $p < 0.05$.

The micronuclei counts before and after the trial were clearly associated ($p < 0.001$), but we observed only a modest correlation in both the placebo group (Pearson R = 0.29) and the β-carotene group (Pearson R = 0.40). The micronuclei counts thus show a large within-person variation (see also figure 2).

Discussion

This trial in heavy smokers shows a reduction in frequency of micronucleated sputum cells *after supplementation with* β-carotene, suggesting that the inverse epidemiological association between β-carotene and lung cancer[3,4,5] is indeed due to β-carotene, and not to associated food or life-style factors. These results thus support a protective role for β-carotene in the development of human cancer, as proposed by Peto et al. (1984).[21] Moreover, the results indicate that β-carotene is protective in man by preventing DNA damage in target tissues, thus providing a plausible mechanism of action.

The approximately 30% reduction in micronuclei after β-carotene treatment is in accordance with the effect of β-carotene reported in buccal mucosa of betel nut chewers[10] and tobacco chewers.[12,13] Our findings extend these observations to cigarette smoke-induced tracheobronchial micronuclei, which may reflect lung cancer risk.[22,23] Moreover, as plasma retinol levels were not changed, this study shows that the provitamin β-carotene does not exert its action after intestinal or hepatic conversion to retinol. It thus seems that β-carotene *per se* is effective at the cellular level. The protective action of β-carotene may be explained by its antioxidant capacity to quench highly reactive singlet oxygen and free radical species.[24] Free radicals are abundant in cigarette smoke and tar[25] and are believed to initiate cancer by damaging DNA.[26] In addition, β-carotene has been hypothesized to be effective after conversion to retinol at a tissue or cellular level.[27] β-carotene could thus rapidly compensate for local deficiencies in retinol, which may be induced by carcinogens.[28]

Micronuclei in exfoliated epithelial cells reflect the extent of chromosome breakage due to carcinogenic exposure, when the cells were dividing a few days or weeks earlier, in the

basal layer of the epithelium of the tracheobronchial tree.[22] As DNA damage is considered crucial in carcinogenesis,[8] the frequency of micronuclei may thus reflect cancer risk. In several experimental models, including the rat bronchial carcinoma model,[22,23] high frequencies of micronuclei are observed after carcinogen exposure. In man, numbers of micronuclei in buccal mucosa cells have been found to increase after exposure to tobacco and alcohol,[29] betel quid[30] and X-radiation;[31] all these exposures are known causes of oral cancer. Similarly, smokers have elevated frequencies of micronucleated cells in expectorated sputum[5] and bronchial brushings.[9] These observations strongly suggest that micronuclei indeed reflect early or intermediate stages of the carcinogenic process. Follow-up studies on the predictive value of micronuclei for cancer development, however, have not been published.

Surprisingly, our data show higher initial micronuclei counts in the β-carotene group, indicating unsuccessful randomization. The number of inevaluable volunteers, as well as the reasons for inevaluability were similar in the placebo and the β-carotene groups, and can therefore not explain this difference. During the trial, the β-carotene and placebo groups were equally represented in all staining and scoring runs, so that any systematic difference in staining or scoring procedures between the two groups seems improbable. Moreover, all slides were coded, and scored by a single technician, and all other baseline characteristics measured (table 1) were comparable between the two groups. Regression to the mean may have influenced the observed reduction in the β-carotene group, but cannot have influenced after-treatment micronuclei counts. Despite the higher initial count, the after treatment counts were significantly lower in the β-carotene group, even without adjustment for initial counts.

In the treatment group, we did not observe a dose-response relationship between plasma β-carotene and reduction in micronuclei count. However, we evaluated the effect of only one high dose of β-carotene, and almost all subjects showed dramatic increases in plasma levels. Furthermore, the limited number of subjects, the low frequency of micronuclei, as well as the considerable within-person variation in micronuclei counts make it difficult to evaluate a dose-response relationship. The low frequency of micronuclei in this study may be partly due to our stringent scoring criteria, aimed at identifying micronuclei reproducibly and with a high certainty. The large within-person variability may be explained by an inherent variability in sampling site, as expectorated cells may originate from all sites in the tracheobronchial tree. This large random sampling variation implies that the statistical power of studies using sputum is only sufficient to demonstrate large effects in study groups of considerable size. For future studies, repeated sampling and scoring can be used to diminish within-person variation. In addition, a run-in period prior to treatment could be used to assess eligibility with respect to sputum production and to stratify the treatment groups on micronuclei counts. Alternatively, studies using bronchial brushings, though more invasive, have the merit of being site-specific and may prove more useful to evaluate smaller effects, such as dose responses. Such studies may also be used to evaluate variations in counts between different sites. In addition, cellular levels of

β-carotene could be studied in future studies, since plasma β-carotene levels may not wholly reflect tissue levels of β-carotene in the tracheobronchial tree.

Our data suggest that β-carotene is effective by preventing DNA damage and may thus affect early or intermediate stages of carcinogenesis. This is in line with laboratory studies that indicate a role for β-carotene in antimutagenesis and prevention of malignant transformation.[24] However, there are also indications that β-carotene may affect later stages of carcinogenesis.[32] The recently reported lack of effect of β-carotene in trials on cervical dysplasia[33] and second skin cancers[34] may, apart from site specificity, be explained if β-carotene is primarily effective in earlier stages of carcinogenesis. To address this question, the ongoing intervention studies in cancer incidence will need a long follow-up. Indeed, the β-carotene trial in the Physicians Health Study has recently been extended to cover more than 10 years follow-up.[35]

This study yields evidence that the observed inverse association between β-carotene and lung cancer is due to β-carotene *per se*. Though the predictive value of micronuclei for cancer risk remains to be shown definitively, our results suggest that β-carotene may indeed reduce human cancer risk. It is clear that the health benefits of stopping smoking will far outweigh those of dietary changes.[4] These results should therefore not be explained as a way to prevent lung cancer in people who continue to smoke.

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Effect of beta-carotene on immunological indices in healthy male smokers

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Abstract

To evaluate the immunomodulatory effects of β-carotene we performed a randomized, double-blind trial in healthy male cigarette smokers. Lymphocyte subsets in peripheral blood were assessed by using double labeling with monoclonal antibodies before and after 14-weeks β-carotene (20 mg/day; n = 21) or placebo (n = 24) supplements. In addition we measured the *ex vivo* phytohemagglutinin and concavalin A induced lymphocyte proliferation in a separate group (23 placebo, 24 β-carotene). The β-carotene and placebo groups were comparable on all initial characteristics. During the intervention plasma concentrations of β-carotene increased 13-fold in the treatment groups whereas retinol concentrations remained constant. β-carotene had no effect on lymphocyte subpopulations in peripheral blood. After treatment the β-carotene group showed 12% higher PHA-induced lymphocyte proliferations than the placebo group (p = 0.02). For ConA induced proliferations no significant difference was observed. These results suggest that supplementary β-carotene can moderately enhance certain aspects of immune response in healthy male cigarette smokers.

Introduction

Within the past few years there has been an increasing interest in the potential of β-carotene to provide protection against human cancers.[1,2] Epidemiological studies have reported inverse relationships between plasma or dietary β-carotene and the incidence of cancer at several sites, especially the lung.[3,4] The putative cancer preventing potential of β-carotene may be explained by its antioxidant capacity to quench free radicals, thus preventing DNA damage and neoplastic transformation.[5] In addition, immunomodulatory effects of β-carotene have been proposed as a mechanism for tumor prevention and several animal as well as human studies have shown immunomodulatory effects of β-carotene.[6]

Immunological studies in humans on the effect of β-carotene so far have focused on assessment of lymphocyte subsets in peripheral blood and these studies have yielded inconsistent results. Smaller studies in elderly subjects[7] and in patients with premalignant lesions[8] have demonstrated positive effects of β-carotene supplementation, whereas a larger

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study in healthy volunteers[9] failed to demonstrate any effect. Possibly, effects of β-carotene are only found in subjects with suboptimal immune or β-carotene status. Moreover, these studies are difficult to interpret as alterations in lymphocyte subsets may only have limited implications for the effectiveness of the immune response *in vivo*.

For further elucidation of the involvement of β-carotene in human immune function, measurements of functional indexes of immune response may be more informative than quantitation of lymphocyte subsets. In addition, studies in humans with low β-carotene status seem warranted. Cigarette smokers are known to have a marginal β-carotene status,[10] and the epidemiological evidence for a cancer preventive potential of β-carotene is especially consistent for smoking-induced lung cancer.[3,4] We therefore performed a randomized, double-blind, placebo-controlled trial in healthy male smokers. In addition to quantitation of lymphocyte subsets, we assessed the influence of β-carotene supplementation on mitogen-induced lymphocyte proliferative response.

Subjects and methods

Study design

For this study, immunological indexes were assessed in two separate subgroups of subjects who participated in an intervention trial on β-carotene and biomarkers for cancer risk.[11] Healthy male employees of the AMEV Insurance Company, the Taxation Office, and the Power Company at Utrecht, the Netherlands were asked to volunteer for the intervention trial, which was approved by an External Review Board for experiments with human volunteers. All participants (total n = 163) had smoked ≥ 15 cigarettes/day for > 2 years, did not use preparations containing retinol or carotenoids, and did not report exposure to xenobiotic chemicals during working or leisure time. The volunteers were prestratified by age, duration, and quantity of smoking and randomly assigned to either β-carotene (20 mg capsules, Hoffmann – La Roche Ltd, Basel, Switzerland) or placebo treatment.

Blood samples were collected before and after the 14-weeks treatment. The participants were instructed to take capsules daily with the evening meal: 2 capsules per day during the first 2-weeks, followed by 1 capsule/day for the next 12-weeks. Every 4-weeks, the participants were sent their next packet of 28 capsules and were asked to return the used strips with the remaining capsules to monitor compliance. In addition, β-carotene was determined in a blood sample taken after 7-weeks treatment.

Lymphocyte subsets were initially assessed in a subset of 52 participants (26 placebo, 26 β-carotene). Of these 52 participants, 5 subjects (2 placebo, 3 β-carotene) withdrew during the trial. One β-carotene participant was omitted from the data analysis because his plasma β-carotene concentrations did not increase. For this subject, pill counts to evaluate compliance were not available. In addition, one β-carotene participant was unable to donate blood on the scheduled final day, leaving 45 subjects (24 placebo, 21 β-carotene) for analysis of lymphocyte-subset data. To minimize influences of between-run variations, placebo and β-carotene subjects for the lymphocyte subset study were equally divided over

four initial and six final measurement days.

Lymphocyte stimulation tests were initially performed in a subset of 51 participants (25 placebo, 26 β-carotene). Of these 51 participants, one placebo and one β-carotene subject withdrew during the trial. In addition, one placebo and one β-carotene participant were not able to donate blood on the scheduled final day, leaving 47 subjects (23 placebo, 24 β-carotene) for analysis of the lymphocyte proliferation data. For the lymphocyte proliferation study, placebo and β-carotene subjects were equally divided over six initial and five final measurement days.

Lymphocyte subsets

Quantitation of lymphocyte subsets was performed by double labeling in whole blood with potassium EDTA as an anticoagulant. Within 3 hours after venipuncture, 100 μl blood was incubated for 15 min in the dark at room temperature with one of the following combinations of monoclonal antibodies. Red blood cells were lysed by addition of 2 ml FACS-lysing solution (Beckton Dickinson, San Jose, CA) and subsequent incubation in the dark during 10 min at room temperature. After centrifugation (5 min, 400 x g) the cell pellet was washed with 3 ml of phosphate-buffered saline. The cells were then immediately fixed in 0.5 ml 0.5% paraformaldehyde solution and stored at 4°C until flowcytometric analysis on the following day. The samples were measured on a FACStar plus (Beckton Dickinson). The following combinations of monoclonal antibodies were used: CD4(leu-3a) fluorescein isothiocyanate (FITC) conjugated/CD8(leu-2a) phytocrythin (PE) conjugated, for the detection of T helper/inducer (CD4+/CD8-) and T suppressor/ cytotoxic (CD8+/CD4-) cells. CD3(leu-4)FITC conjugated/human leukocyte antigen (HLA)-DR PE conjugated, for the detection of mature T cells (CD3+/HLA-DR-) and activated T cells (CD3+/HLA-DR+). CD3(leu-4)FITC conjugated/CD16(leu-11c) and CD56(leu-19) PE conjugated, for the detection of non-major histocompatibility complex (MHC) restricted cytotoxic T cells (CD3+/CD16 and 56+) and natural killer (NK) cells (CD3-/CD16 & 56+). CD45RA(leu-18) FITC conjugated/CD4(leu-3a) PE conjugated, for the detection of T naive (CD4+/CD45RA+) and T memory (CD4+/CD45RA-) cells. CD2(leu-5b) FITC conjugated/CD19(leu-12) PE conjugated, for the detection of T (CD2+/CD19-)) and B (CD2-/CD19+) cells. All monoclonal antibodies were purchased from Beckton Dickinson.

Lymphocyte stimulation tests

Within 3 hours after venipuncture, mononuclear cells (MNC) were separated from heparinized whole blood by means of a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient.[12] A volume of 200-μl was placed in a flat-bottom microtiter well (Nunc, Roskild, Denmark) containing 10^5 MNC and mitogen in RPMI-1640 with 2 mmol/l glutamine/0.5 g/l gentamycin, and 0.25 mg/l Fungizone (Flow, Irvine, Scotland). The culture medium was supplemented with either 20% fetal calf serum (FCS) (Flow) or 20% autologous heparin plasma, which was freshly prepared from the same blood sample that was used for MNC separation. The mitogen concentrations were 20, 50 and 80 mg/l for phytohemagglutinin

(PHA) (Wellcome, Dartford, England) and 5, 20 and 30 mg/l for concanavalin A (ConA) (Sigma, St Louis, MO). The microtiter plates were incubated during 96 hours at 37°C and 5% CO_2. Detection of cell proliferation was performed by using the MTT (3-4,5 dimethylthiazol-2-yl-2,5-diphenyl tetrazoliumbromide) method described first by Mosmann.[13] MTT (20 μl 5 g/l, Sigma) was added 6 hours before the end of the incubation period. The formed crystals were dissolved by addition of a 25% sodium-dodecyl-sulphate solution. Increases in optical density at 550 nm are a measure for increases in cell number. Increases in cell numbers were only measured once, after 96 hours culture.

Blood parameters

After venipuncture, nonfasting blood samples containing sodium EDTA as anticoagulant were stored overnight in the dark at 4°C for 20-23 hours. Immediately after opening the evacuated tubes, the sum of L-ascorbic + dehydro-L-ascorbic acid (vitamin C) was assessed in whole blood by HPLC with fluorimetric detection.[14] All-*trans* retinol, α-tocopherol, β-carotene, and total carotenoids were assayed in plasma (stored at −80°C) by HPLC with colorimetric detection.[15] Plasma cotinine concentrations were determined by gas chromatography.[16]

Data analysis

Initial baseline values and changes in these values during the intervention period were compared between the placebo group and the β-carotene group using the unpaired t-test. We used multiple-linear regression to quantify differences in final values between the placebo and treatment group after adjustment for initial values and residual confounding. Analyses of covariance was used for statistical testing. All data were analysed by using the BMDP package.[17] Two-sided p values < 0.05 were considered statistically significant.

Results

Lymphocyte subset study

Table 1 shows that the placebo and β-carotene group in the lymphocyte subset study are comparable for all characteristics, and that except for plasma concentrations of β-carotene, only minor changes occurred during the intervention trial. After 7-weeks of supplementation mean plasma β-carotene had increased 13-fold in the β-carotene group (from 0.30 to 3.94 μmol/l) and this increase remained stable until the end of the trial (table 1). Typical chromatograms from a β-carotene treated and a control subject are shown in figure 1. The minimum increase in plasma β-carotene was 3.3-fold. Pill counts showed that a mean of 91% of all capsules were taken (data for 40 subjects). Skin yellowing was reported by three supplemented subjects. Three subjects reported taking vitamin supplements (one from the placebo, two from the β-carotene group); they occasionally took vitamin C. Their blood vitamin C concentration, however, were not very high (12.6, 63.5, and 43.8 μmol/l, respectively).

Table 1. Baseline characteristics (SD) and changes in these characteristics during a 14-weeks trial on lymphocyte subsets in male smokers, assigned to either β-carotene (20 mg/day) or placebo treatment*

	Placebo group (n = 24)		β-carotene group (n = 21)	
	Baseline	Change	Baseline	Change
Age (yrs)	39.1 ± 9.1	n.a.	39.8 ± 8.9	n.a.
Cigarettes/day	20.8 ± 5.7	n.a.	20.8 ± 4.5	n.a.
Blood vitamin C (μmol/l)	29.8 ± 14.8	4.8 ± 11.7	32.9 ± 15.3	−0.3 ± 9.4
Plasma retinol (μmol/l)	2.36 ± 0.85	−0.08 ± 0.49	2.49 ± 0.52	0.09 ± 0.41
Plasma α-tocopherol (μmol/l)	29.5 ± 6.4	1.8 ± 4.3	32.2 ± 7.5	1.8 ± 3.7
Plasma β-carotene (μmol/l)	0.33 ± 0.22	−0.02 ± 0.16	0.30 ± 0.14	3.64 ± 1.99*
Plasma cotinine (μg/l)	311 ± 116	−11 ± 64	316 ± 121	0 ± 75
Lymphocyte count (x10^9/l)	2.14 ± 0.39	−0.01 ± 0.35	2.11 ± 0.63	0.05 ± 0.44

* $\bar{x}$ ± SD. n.a. = non applicable.

† β-carotene group significantly different from placebo groep, $p < 0.001$.

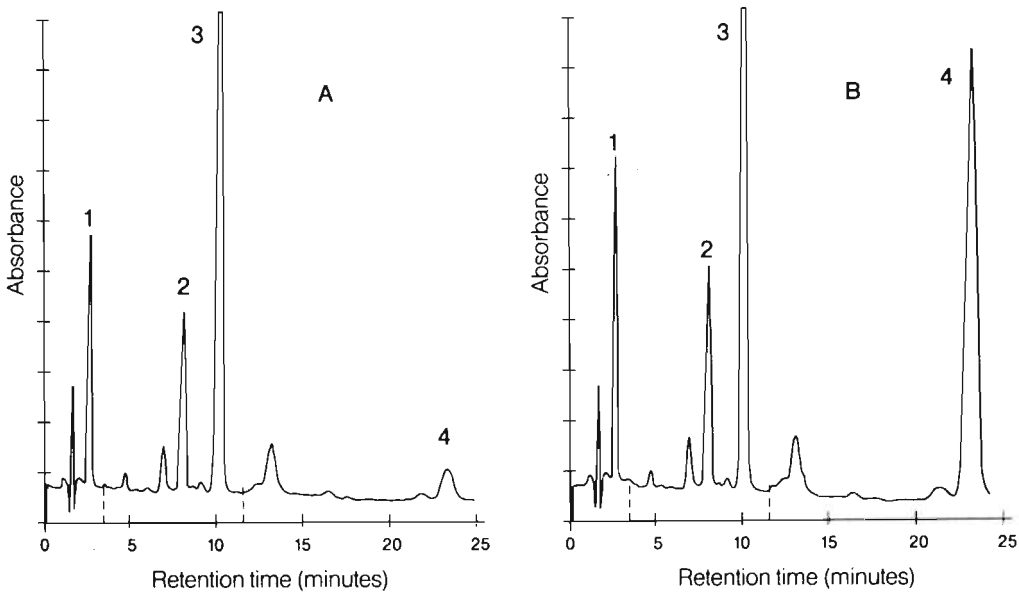

Figure 1. Typical chromatograms from a control subject (A) and a 14-weeks β-carotene treated subject (B). 1) Retinol 2) α-tocopherol 3) Internal standard (tocopheryl acetate) 4) β-carotene. Dotted lines indicate wavelength shifts (325 nm; 292 nm; 445 nm respectively).

The β-carotene and placebo groups showed similar initial values for lymphocyte subsets (table 2). No effect of β-carotene supplementation were observed on the lymphocyte subsets (table 2).

Table 2. Lymphocyte subsets as percentage of total lymphocyte count during a 14-weeks trial in male smokers, assigned to either β-carotene (20 mg/day) or placebo treatment.*

	Placebo group (n = 24)†		β-carotene group (n = 21)†		β-carotene -placebo after study (95% CI)‡
	Baseline	Change	Baseline	Change	
CD2+/CD19- (total T)	80.7 ± 5.0	0.7 ± 4.4	77.9 ± 5.9	2.3 ± 5.1	0.3 (-3.6:4.2)
CD2-/CD19+ (total B)	12.0 ± 4.7	-0.4 ± 3.2	13.9 ± 5.3	-0.3 ± 3.7	1.2 (-0.8:3.2)
CD4+/CD8- (T helper/inducer)	44.3 ± 5.9	0.3 ± 4.7	44.8 ± 7.2	-0.1 ± 6.3	-1.6 (-5.2:2.0)
CD4-/CD8+ (T suppressor/cytotoxic)	33.2 ± 6.8	1.6 ± 3.5	29.8 ± 8.1	0.7 ± 2.6	-1.4 (-3.5:0.8)
CD3+/HLADr- (mature T)	66.2 ± 5.7	-1.9 ± 5.8	65.1 ± 7.2	1.0 ± 4.5	1.8 (-1.8:5.4)
CD3+/HLADr+) (activated T)	7.0 ± 3.4	0.0 ± 4.0	6.5 ± 2.7	0.5 ± 2.9	0.7 (-1.4:2.8)
CD3+/CD16 & 56+ (cytotoxic T)	6.0 ± 6.2	-0.2 ± 2.8	4.3 ± 3.4	0.0 ± 2.9	0.1 (-1.6:1.7)
CD3-/CD16 & 56+ (NK)	14.7 ± 6.6	1.7 ± 6.0	13.5 ± 5.1	-0.3 ± 3.1	-1.8 (-5.2:1.5)
CD45r+/CD4+ (T naive)	17.0 ± 6.9	-0.2 ± 4.2	17.0 ± 6.5	-0.9 ± 3.4	-0.6 (-2.8:1.7)
CD45r-/CD4+ (T memory)	30.3 ± 7.9	0.2 ± 4.9	32.2 ± 7.7	0.3 ± 5.2	0.0 (-2.9:2.9)

* $\bar{x}$ ± SD.

† For some individual measurements data are missing.

‡ Differences in final values after adjustment for initial values, age, blood levels of cotinine and vitamins A,C,E, and variation between measurement days.

Lymphocyte proliferation study

Baseline characteristics of the lymphocyte-proliferation study are given in table 3.

Table 3. Baseline characteristics and changes during a 14-weeks trial on lymphocyte-proliferation respons in male smokers assigned to either β-carotene (20 mg/day) or placebo treatment.*

	Placebo group (n = 23)		β-carotene group (n = 24)	
	Baseline	Change	Baseline	Change
Age (yrs)	40.7 ± 10.9	n.a.	41.1 ± 10.8	n.a.
Cigarettes/day	18.6 ± 3.5	n.a.	19.4 ± 4.5	n.a.
Blood vitamin C (μmol/l)	40.5 ± 17.0	3.12 ± 11.7	41.0 ± 17.4	-3.8 ± 12.3
Plasma retinol (μmol/l)	2.49 ± 0.85	-0.12 ± 0.49	2.55 ± 0.48	-0.03 ± 0.53
Plasma α-tocopherol (μmol/l)	31.4 ± 7.2	1.3 ± 4.3	32.4 ± 7.0	0.1 ± 3.6
Plasma β-carotene (μmol/l)	0.28 ± 0.13	0.01 ± 0.16	0.35 ± 0.16	4.78 ± 2.51*
Plasma cotinine (μg/l)	306 ± 112	-3 ± 64	317 ± 108	-10 ± 75

* $\bar{x}$ ± SD. n.a. = not applicable.

† β-carotene group significantly different from placebo groep, $p < 0.001$.

Here again, the placebo and β-carotene groups are comparable for all characteristics and, except for plasma concentrations of β-carotene, only minor changes occurred during the intervention trial. Mean plasma β-carotene increased 13-fold after 7-weeks supplementation in the β-carotene group and 14-fold by the end of the trial (table 3). A mean of 90% of all capsules were taken (data for 42 subjects) and plasma concentrations of β-carotene increased in all supplemented subjects, the minimum increase being 3.3-fold. Skin yellowing was reported by five supplemented subjects. None of the subjects reported taking vitamin supplements.

Table 4 gives results for lymphocyte proliferation at 80 mg/l PHA and 30 mg/l ConA. These concentrations resulted in maximal proliferation in the autologous plasma cultures. β-carotene supplementation did not have an influence on ConA-induced proliferation either in FCS or in autologous plasma culture. β-carotene supplementation resulted in an increase in PHA-induced proliferation, but only in lymphocyte cultures supplied with autologous plasma. For the cultures with the other concentrations of mitogens, similar data were obtained (results not shown).

Table 4. Lymphocyte proliferation as increases in optimal density after different incubations during a 14-weeks trial in male smokers, assigned to either β-carotene (20 mg/day) or placebo treatment.*

	Placebo group (n = 23)		β-carotene group (n = 24)		β-carotene - placebo after study (95% CI)†
	Baseline	Change	Baseline	Change	
PHA (80 mg/l) plasma	1.12 ± 0.24	0.04 ± 0.28	1.01 ± 0.25	0.22 ± 0.24‡	0.14 (0.02:0.26)
PHA (80 mg/l) FCS[1]	1.05 ± 0.42	0.03 ± 0.29	1.06 ± 0.33	−0.11 ± 0.18	−0.11 (-0.3:0.05)
ConA (30 mg/l) plasma	0.99 ± 0.30	0.01 ± 0.32	1.00 ± 0.28	0.08 ± 0.20	0.08 (-0.06:0.21)
ConA (30 mg/l) FCS[2]	0.66 ± 0.34	−0.05 ± 0.35	0.62 ± 0.52	−0.08 ± 0.57	−0.01 (-0.30:0.29)

* $\bar{x}$ ± SD; PHA, Phytohemagglutinin; ConA, concavalin A; FCS, fetal calf serum.

† Differences in final values after adjustment for initial values, age, blood concentrations of cotinine and vitamins A,C,E, and variation between measurement days.

‡ β-carotene group significantly different from placebo group (p = 0.01).

[1] n = 18 placebo & 17 β-carotene subjects; [2] n = 13 placebo & 17 β-carotene subjects.

To obtain an unbiased estimate of the intervention effect, we calculated the difference between the placebo group and the β-carotene group after intervention, allowing for the small differences that still existed between both groups at the start of the study. After adjustment for age; plasma concentrations of cotinine, vitamins A, C and E; variation between measurement days; and initial proliferation response, the after-treatment proliferation induced by PHA (80 mg/l) was estimated to be 12% higher in the β-carotene group than in the placebo group (1.13 vs. 1.27; p = 0.02).

Discussion

This study in healthy male smokers shows that 14-weeks supplementary β-carotene has an enhancing effect on *ex vivo* proliferative T cell response as induced by the mitogen PHA, whereas marker expression on peripheral blood lymphocytes is not affected by β-carotene.

The lack of effect of β-carotene on lymphocyte subsets in this study can hardly be attributed to an inadequate dose of β-carotene as 20 mg/day is approximately 5–10-fold the normal intake. In addition, we used 40 mg/day for the first 2-weeks to saturate tissue concentrations of β-carotene. Indeed, several participants observed skin discoloration, rendering the study not completely double blind. Plasma concentrations of β-carotene in the supplemented group dramatically increased to concentrations similar to those reported by Ringer et al.[9] after 45 mg/day for 1 month and to those reported by Watson et al.[7] after 30 mg/day for two months. Moreover, we did not observe any effect of β-carotene on lymphocyte subsets after we excluded supplemented subjects with the lowest quartile (< 1.88 μmol/l) of after-treatment β-carotene concentrations (results not shown).

The absence of an effect of β-carotene on marker expression is in accordance with a recently reported study in 50 healthy, non-smoking males and females aged 18 - 54 years, who used doses up to 300 mg/day.[9] Our study thus extends these previous observations to smoking individuals, who are known to have low plasma β-carotene concentrations. In contrast, three other smaller studies in humans did show effects of β-carotene on marker expression. The first of these studies,[18] however, is difficult to compare with ours as the observed increase in CD4+ expression may be attributable to a concomitant increase in plasma retinol, suggesting suboptimal initial vitamin A status. A second, non-placebo-controlled study[8] likewise does not report plasma retinol concentrations, which precludes evaluation of vitamin deficiencies in the studied subjects. Moreover, this second study used patients with precancerous conditions, which may imply underlying immunological changes. The third study[7] reported increases in the percentage of cells with markers for NK, T helper, interleukin 2, and transferrin after 2 months supplementation of 16 subjects with > 30 mg/day. In four subjects receiving 15 mg/day, these increases were not observed despite similar increases in plasma β-carotene. The study, however, did not use double staining, B cells or cell types that decreased were not reported,[19] and the time elapse between venipuncture and quantitation of lymphocyte subsets was not given. In addition, this last study used elderly subjects. Age has been shown to influences the immune system.[20] The discrepancies between the studies may be explained if β-carotene only influences marker expression in people with age- or disease-induced suboptimal immune status.

The increase in PHA-induced lymphocyte proliferation in this study does not seem to be related to a retinol effect of β-carotene, as plasma retinol concentrations remained unchanged during the study. Likewise, concentrations of the antioxidants C and E were not affected. The increase is remarkable, as we did not observe any influence of β-carotene on lymphocyte subpopulations to explain increased mitogenesis. The increased PHA-induced

mitogenesis thus seems to be related to a plasma factor, most probably β-carotene. This suggestion is supported by our observation that PHA-induced mitogenesis was increased in cultures supplied with autologous (β-carotene rich) plasma, but not in cultures supplied with FCS. It is difficult to judge the biological significance of the observed 12% increase in PHA-induced mitogenesis. However, our *ex vivo* assay used only 20% plasma. The *in vivo* proliferative response, in a 100% plasma environment, may therefore be substantially more enhanced after β-carotene supplementation.

The immuno-enhancing mechanism of the antioxidant β-carotene has been hypothesized[6] to involve the quenching of free radicals, which could lower the concentration of immunodepressing lipid peroxides, alter arachidonic acid metabolism, stabilize lysosome membranes, or protect nuclear structures. A nonspecific antioxidant mechanism would imply that the same effect should be seen with all mitogens capable of stimulating lymphocyte proliferation. However, we did not observe a significant effect of β-carotene on ConA-induced proliferation, which could suggest a more specific interaction of β-carotene with lymphocyte functions. Interestingly, Ringer et al.[9] mention (without giving data) mildly augmented PHA-induced mitogenesis after β-carotene supplementation in humans, whereas another mitogen (OTK3) response was not affected. Similarly, Meydani et al.[21] report enhancement of PHA but not ConA mitogenesis after vitamin E supplementation in elderly subjects. A study in rats[22] showed augmented responses to both ConA and PHA after β-carotene treatment, but in this study retinol concentrations were also increased.

It is tempting to extrapolate our data to the evaluation of a cancer-preventive potential of β-carotene. Such interpretations, however, can only be very cautiously made. First, because peripheral blood lymphocytes may only partly reflect the distribution and activities of lymphocytes in organs more relevant to carcinogenesis.[23] For instance, it is notable that lymphocyte subsets in broncheo-alveolar lavage differ from those in peripheral blood.[24] Second, because the T cell responses studied may have only limited relevance in immunomodulation of carcinogenesis. Several forms of non-T cell mediated immunity may also be important.[25] Though we did not observe an effect of β-carotene on the number of peripheral NK cells (CD3-/16 and 56+), an effect on NK activity may exist. Finally, it should be noted that a substantial number of human tumors do not exhibit any immunogenic properties.[26] The concept of immune surveillance in the regulation of carcinogenesis may therefore only apply to certain forms of cancer.

We conclude that this study yields evidence that supplementary β-carotene can moderately enhance certain aspects of immune response in healthy male smokers. The significance of this finding for the putative cancer-preventive potential of β-carotene remains to be established. In our study's group of smokers had marginal β-carotene status, we did not observe an effect of β-carotene on marker expression in peripheral lymphocytes. However, it cannot be excluded that β-carotene may exhibit cancer preventive properties by modifying marker expression in subjects with suboptimal immune status.

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The effect of beta-carotene on sputum cytology in smokers: a preliminary study

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Abstract

A large body of evidence suggests that β-carotene may reduce lung cancer risk. Since the use of biomarkers may be a useful approach to human cancer risk, we performed a randomized blinded controlled trial in smokers. This allowed a preliminary evaluation of the effect of 14-weeks β-carotene (20 mg/day) on bronchial metaplasia in sputum cytology as a marker for potential premalignancy. In addition, we evaluated determinants of sputum production in healthy asymptomatic smokers.

Initial metaplasia scores were somewhat higher in the β-carotene group (n = 33) than in the placebo group (n = 42) (p = 0.06). Plasma β-carotene levels increased 14-fold during treatment. Final metaplasia scores were similar in both groups (p = 0.69), and there was no decrease in metaplasia scores in the β-carotene group (p = 0.75). We observed no significant correlation between initial and final metaplasia scores in either the β-carotene (Spearman R = 0.18, p = 0.3) or placebo group (Spearman R = 0.17, p = 0.3).

Of the 150 potential participants in this trial 75 were not eligible because they failed to produce sputum samples (n = 29), or because samples were unsatisfactory (n = 46). The eligible group was older (41 vs. 37 years) and had smoked longer (23 vs. 19 years), but had similar cigarette consumption (mean 21/day) and plasma cotinine levels.

This study provides preliminary evidence that β-carotene has no influence on minor bronchial metaplastic changes as detected in sputum cytology. Moreover, the results indicate that the performance of future studies can be increased by repeated sampling or use of brushings to diminish variations in cytology, by increasing size and time span, and by pre-selection of subjects with more severe lesions. Therefore, cautious interpretation of the absence of an effect of β-carotene is warranted.

Introduction

Several lines of evidence indicate that β-carotene may reduce lung cancer risk. Epidemiological studies have been remarkably consistent in reporting an association between high consumption of β-carotene-rich fruits and vegetables and lower risk of lung cancer.[1,2] Experimental studies indicate that β-carotene may be protective by providing antioxidant

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protection against free radical induced DNA- and cell-damage.[2,3] In addition, its provitamin A activity may imply a beneficial effect on cellular differentiation and proliferation.[2,3]

To test the β-carotene - lung cancer hypothesis, several human intervention studies have been initiated. A limited number of these studies use cancer incidence as an endpoint and are necessarily large scale and lengthy.[2,4,5] Several other studies focus on intermediate biomarkers for cancer risk.[2,6] Though these studies cannot provide definite answers, they do have the merit of providing insight in mechanisms within an efficient study size and time schedule.[7] Also, these studies yield valuable information for planning and evaluating large scale trials on cancer incidence.[7]

We have previously conducted a double-blind placebo-controlled trial to evaluate the effect of β-carotene on markers for DNA damage in smokers.[8,9] Whereas markers for DNA damage may reflect initiation and earlier stages in carcinogenesis, other markers may bear relevance on later stages of carcinogenesis. A promising marker in this respect is degree of bronchial metaplasia observed in cytological evaluation of expectorated sputum.[10,11] Since sputum samples were routinely collected in the previously conducted study, we performed a preliminary evaluation of the effect of β-carotene on bronchial metaplasia in sputum samples. In addition, since methodological information on this marker is scarce, we studied determinants of spontaneous sputum production in healthy asymptomatic smokers and variation in bronchial metaplasia scoring over a 14-weeks period.

Subjects and methods

Study design

Healthy male employees of the AMEV Insurance Company, the Taxation Office and the Power Company at Utrecht, the Netherlands were asked to volunteer for the intervention trial, which was approved by an External Review Board for experiments with human volunteers. All participants had smoked at least 15 cigarettes per day for over 2 years, did not use preparations containing retinol or carotenoids, and did not report exposure to chemicals during working or leisure time. The volunteers were prestratified by age, duration and quantity of smoking and randomly assigned to either β-carotene (20 mg capsules, F. Hoffmann - La Roche Ltd.) or placebo treatment.

Blood and sputum samples were collected before and after the 14-weeks treatment. The participants were instructed to take capsules daily with the evening meal; two capsules per day during the first 2-weeks, followed by one capsule per day for the next 12-weeks. Each 4-weeks, the participants were sent their next strip of 28 capsules, and were asked to return the used strips with the non-taken capsules to monitor compliance. In addition, β-carotene was determined in a blood sample taken after 7-weeks treatment.

Initially, 163 smokers volunteered to participate; 83 were assigned to placebo treatment, 80 were assigned to β-carotene treatment. During the trial, a total of 13 smokers (6 placebo, 7 β-carotene) withdrew their participation because of stopping smoking (n = 4), illness or accident (n = 3), private circumstances (n = 1), forgetting to take capsules (n = 2), or without

giving a reason (n = 3). Of the 150 smokers who completed the trial, 75 (35 placebo, 40 β-carotene) were not eligible for this study on sputum cytology; 29 failed to spontaneously produce sputum samples, whereas samples were unsatisfactory in another 46 subjects. This left 75 subjects (42 placebo, 33 β-carotene) for the study of the effect of β-carotene on sputum cytology.

Sputum cytology

Sputum was collected and processed as described in detail by Saccomano et al.[12] Each participant received a careful individual instruction on how to produce a specimen from 'deep in the lungs'. Sputum was collected at home on three consecutive mornings, directly after rising and after carefully rinsing the mouth. The three, or minimally two samples, collected in preservative (50 ml 50% ethanol with 2% polyethylene glycol (Carbowax 1540, Merck)), were mixed, homogenized, centrifuged and smeared on slides. The slides were stained using the Papanicolaou technique[13] and all slides were assessed by a single cytotechnologist (TH). For each subject, 2 slides were screened and scored according to the standard criteria of the International Histological Classification of Tumors.[14] Based on this classification, a standardized protocol for worsening grades of metaplasia was devised, as follows: (1) Normal cytology (2) Mild metaplasia without atypia (3). Metaplasia without atypia (4) Metaplasia with slight atypia (5) Slight dysplasia (6) Moderate dysplasia (7) Severe dysplasia.

Specimens were considered of unsatisfactory quality if they contained inflammatory elements sufficient to obscure the cells to be examined, or if they contained no alveolar macrophages, indicating that the specimen most likely did not originate from the lower respiratory tract.

Blinded rescoring for 13 samples was performed to evaluate within-observer variability. Agreement was exact in 54% of the samples, whereas agreement was in the same or an adjacent category in 93% of the samples. In addition, a chief cytotechnologist (AvA) performed a blinded rescoring of slides from 23 subjects to evaluate variability between scorers. Agreement between the two observers was 39 % for full agreement and 95% for agreement within the same or an adjacent category.

Blood parameters

Directly after venapuncture, non fasting blood samples containing NaEDTA as anti-coagulant were stored overnight in the dark at 4°C for 20-23 hours. Directly after opening the evacuated tubes, the sum of L-ascorbic + dehydro-L-ascorbic acid (vitamin C) was assessed in whole-blood by HPLC with fluorimetric detection.[15] All-*trans* retinol, α-tocopherol, β-carotene and total carotenoids were assayed in plasma (stored at −80°C) by HPLC with colorimetric detection.[16] Plasma cotinine levels were determined by gas chromatography.[17]

Data analysis

Initial values, final values, and changes in these values during the intervention were compared between groups using the unpaired Student's t-test and the Mann Whitney rank sum test. Spearman rank correlations were calculated to study associations between metaplasia scores

and baseline characteristics. The Wilcoxon signed rank test was used to test changes in metaplasia scores within groups. The Chi square test was used to compare improvement in scores between the placebo and β-carotene group. All statistics were calculated using the BMDP package.[18]

Results

Table 1 shows that the β-carotene and placebo group are comparable on all characteristics and that, except for plasma β-carotene, only minor changes occurred during the intervention trial. After 7-weeks, mean plasma β-carotene levels in the intervention group had increased 14-fold to 4.46 μmol/l, and remained stable up to the end of the trial. Pill counts showed that 93% of all capsules were taken (data for 68 subjects). All participants but one reported to have taken more than 70% of the capsules.

Table 1. Characteristics (mean ± sd) during a 14-weeks intervention trial in male smokers, assigned to either β-carotene or placebo treatment.

	Placebo group (n = 42)		β-carotene group (n = 33)	
	Initial values	Final values	Initial values	Final values
Age (years)	41.9 ± 10.3	n.a.[2]	40.8 ± 9.7	n.a.
Number of cigarettes per day	20.5 ± 6.3	n.a.	23.0 ± 7.7	n.a.
Years of smoking	23.1 ± 11.0	n.a.	23.3 ± 9.4	n.a.
Blood vitamin C (μmol/l)[1]	39.2 ± 19.4	38.4 ± 17.4	38.9 ± 20.2	34.6 ± 15.8
Plasma retinol (μmol/l)	2.32 ± 0.68	2.33 ± 0.56	2.44 ± 0.63	2.53 ± 0.65
Plasma α-tocopherol (μmol/l)	30.5 ± 7.3	32.4 ± 7.1	31.6 ± 7.1	32.6 ± 6.1
Plasma β-carotene (μmol/l)	0.31 ± 0.20	0.27 ± 0.15	0.32 ± 0.13	4.20 ± 2.0*
Plasma total carotenoids (μmol/l)	1.59 ± 0.56	1.66 ± 0.67	1.58 ± 0.58	5.27 ± 2.25*
Plasma cotinine (ng/ml)	316 ± 107	315 ± 131	330 ± 123	322 ± 119

[1] one β-carotene participant has a missing value.

[2] n.a. = not applicable.

* significantly different from placebo group, $p < 0.0001$.

At baseline, metaplasia scores were somewhat, though not significantly, higher in the β-carotene group (figure 1; $p = 0.06$). The worst grade of metaplasia, observed in only one participant, was mild dysplasia. Initial and final metaplasia scores did not differ in the β-carotene group ($p = 0.75$). In the placebo group final metaplasia scores were somewhat higher than initial scores ($p = 0.15$). Final metaplasia scores were similar in both groups (figure 2; $p = 0.69$).

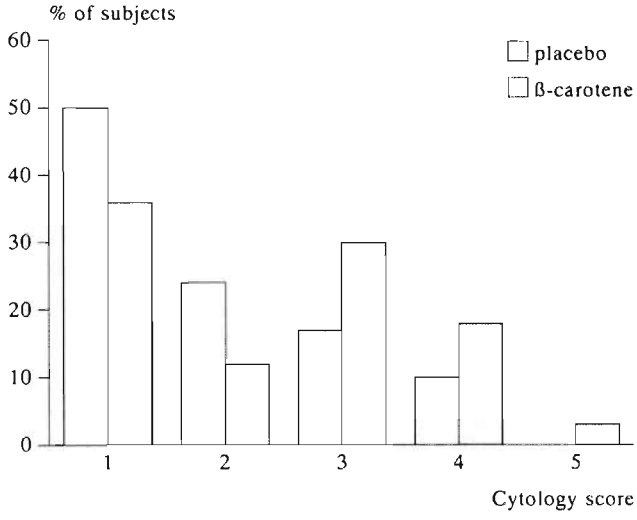

Figure 1 Distribution of cytology scores at the start of a 14-weeks trial in male smokers, assigned to either placebo (n = 42) or β-carotene treatment (n = 33).

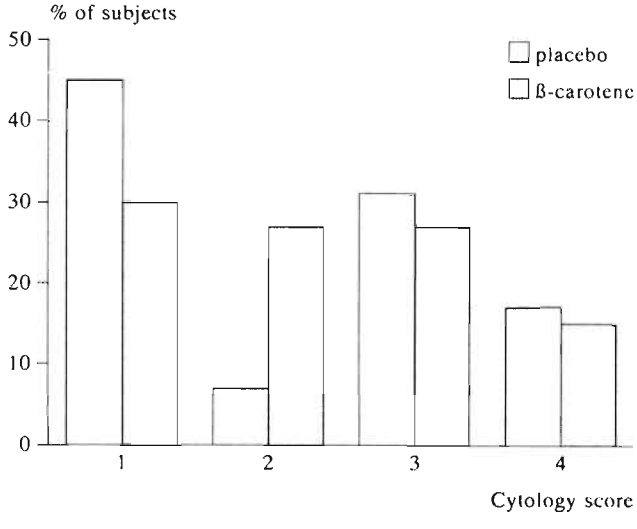

Figure 2 Distribution of cytology scores at the end of a 14-weeks trial in male smokers, assigned to either placebo (n = 42) or β-carotene treatment (n = 33).

Table 2 and 3 shows the relation between initial and final metaplasia scores in both treatment groups. During the intervention, 39% of the carotene group showed improvement in metaplasia scores, compared with 24% in the placebo group (p = 0.16). There was no correlation between the initial and final metaplasia scores either in the β-carotene group (R = 0.18, p = 0.3), or the placebo group (R = 0.17, p = 0.3), or in both groups combined (R = 0.18, p = 0.13). There was no correlation between any of the baseline characteristics in table 1 and the baseline metaplasia scores (all R < 0.20).

Table 2. Numbers of subjects in the placebo group with initial and final cytology scores.

	Initial cytology score			
Final cytology score	1 (n = 21)	2 (n = 10)	3 (n = 7)	4 (n = 4)
1 (n = 19)	12	3	3	1
2 (n = 3)	1	0	1	1
3 (n = 13)	5	5	2	1
4 (n = 7)	3	2	1	1

Table 3. Numbers of subjects in β-carotene group with initial and final cytology scores.

	Initial cytology score				
Final cytology score	1 (n = 12)	2 (n = 4)	3 (n = 10)	4 (n = 6)	5 (n = 1)
1 (n = 10)	3	3	2	1	1
2 (n = 9)	5	0	4	0	0
3 (n = 9)	3	1	3	2	0
4 (n = 5)	1	0	1	3	0

Table 4 compares characteristics of the study participants with those of the group who was not eligible because they produced no or unsatisfactory sputum samples. The study group is older and has a longer smoking history. All other characteristics including daily cigarette consumption and plasma cotinine were similar. Also, compliance was similar in both groups (93% vs. 90% of all capsules taken).

Table 4. Baseline characteristics of study participants compared with non-eligible subjects.

	Study participants (n = 75)	Non-eligible subjects (n = 75)
Age (years)	37.4 ± 9.3	41.4 ± 10.0*
Cigarettes per day	21.1 ± 6.8	21.6 ± 7.0
Years of smoking	19.4 ± 9.7	23.2 ± 10.3*
Blood vitamin C (μmol/l)	34.4 ± 15.9	39.1 ± 19.6
Plasma retinol (μmol/l)	2.31 ± 0.42	2.38 ± 0.66
Plasma α-tocopherol (μmol/l)	30.7 ± 5.7	31.0 ± 7.2
Plasma β-carotene (μmol/l)	0.31 ± 0.18	0.31 ± 0.17
Plasma total carotenes (μmol/l)	1.54 ± 0.62	1.58 ± 0.57
Plasma cotinine (ng/ml)	333 ± 118	322 ± 114

* $p < 0.005$.

Discussion

This study shows no effect of β-carotene on the degree of bronchial metaplasie in heavy smokers. Bronchial metaplasia may be a potential marker of pre-malignancy, since the development of carcinoma of the lung has been considered to proceed along the several increasing stages of atypical metaplasia.[19] Therefore, our results do not seem to lend support to the hypothesis that β-carotene may protect against lung cancer risk in smokers. Several precautions, however, should be considered when interpreting our results.

First, it should be realized that our study was not specifically designed to test a hypothesis with regard to bronchial metaplasia. Sputum samples were routinely collected as part of a trial to evaluate the effect of β-carotene on several markers for DNA-damage in smokers.[8,9] We therefore did not perform a pre-screening, e.g. to select subjects producing satisfactory sputum samples or subjects showing metaplasia on several sampling occasions. As a result the sample size in our study is rather moderate, which precludes the drawing of definitive conclusions. However, Heimburger et al.[10] have reported a similar study in only 37 smokers supplemented with folate and B-12, and 36 placebo subjects. These numbers were sufficient to provide preliminary evidence for a beneficial effect. In contrast, our results do not even provide a tendency towards a positive result since there was essentially no change in average metaplasia scores in the treatment group, and no difference in after-treatment metaplasia scores between the placebo and the β-carotene group.

Though our trial was not specifically designed to study metaplasia, the lack of effect of β-carotene can hardly be attributed to methodological shortcomings, since the trial was fully randomized and placebo controlled and compliance was very good. Table 1 shows, that randomization was successful, even though the subjects were randomized without knowing whether they would be eligible for a cytology study. Moreover, the eligible and non eligible group are very similar, (table 2) with the exception of a difference in age and duration of

smoking. Therefore, a selection bias influencing our conclusions seems improbable. The only point at which randomization was not completely successful, was the average degree of initial metaplasia since the placebo group contained more lower initial cytology scores (figure 1), though not statistically significant. Regression to the mean following the 'accidentally' lower initial scores probably explains the insignificant increase in average metaplasia scores in the placebo group during the trial. It therefore does not seem justified to compare this increase with the 'unchanged' metaplasia scores in the β-carotene group, more so because initial and final metaplasia scores showed no significant correlation. Initial scores thus have little value in predicting final scores, due to large variations over time.

Large variations in cytology scores over time have been previously observed.[10,11] In our study, observer variations will have had some, but not a major contribution to this variation over time, since both inter- and intra-observer agreement upon repeated scoring were satisfactory. Degrees of observer agreement comparable to ours have been reported by Heimburger,[10] whereas somewhat better agreement was reported by Browman et al.[20] In this respect, it is noteworthy that our classification would be expected to yield less exact agreement since we use 5 categories where the other studies use only three. Though we used three pooled samples, sampling variation thus seems to have a major influence on the variation in sputum cytology scores over time. This sampling variation may be inherent to the use of sputum, since samples may originate from all locations in the tracheobronchial tree. Our results thus suggest that interpretations of sputum cytology scores are only useful at group level, and that interpretation of changes in individual scores over time is not warranted. Repeated sputum sampling and scoring may reduce this within person variation. Alternatively, studies using bronchial bushings or biopsies are more invasive but can be expected to be more site specific and yield less variations.

Possibly, our study may have been to short to demonstrate an effect, or the dose of β-carotene may have been insufficient. Though the total cellular turnover of the tracheobronchial epithelium is 30 days,[12] the time span that is relevant for progression or regression is uncertain. However, Heimburger's study demonstrated effects within a four months period. As for the dose of β-carotene, this is equivalent to 5 to 10 times the normal intake, and plasma levels dramatically increased. Other studies in humans have used higher doses (50-300 mg/day) but report similar after-treatment plasma β-carotene levels.[21,22] This suggests that our 20 mg/day dose is sufficient for a maximal increase of β-carotene in plasma and tissue. The dose in our study is similar to the doses used in two large scale cancer-prevention studies.[4,5]

With regard to metaplastic changes, a retinol-mediated effect of β-carotene, possibly through conversion at tissue level[23] seems more plausible than an antioxidant mediated effect. Retinoids have an important role in cellular differentiation and proliferation.[24] The maintenance of a normal bronchial epithelial pattern is partially dependent upon vitamin A,[24] and vitamin A deficiency can induce metaplasia.[25] In this respect it is noteworthy that Arnold et al.[11] recently demonstrated no effect of a synthetic retinoid on sputum cytology in a carefully designed placebo-controlled trial in 150 smokers. Arnolds study[11] thus did not substantiate

preliminary findings from a previous smaller and uncontrolled trial[26] and seems in accordance with our observation of no effect of the pro-vitamin A β-carotene.

Our study cannot exclude an effect of β-carotene on more severe stages of bronchial atypia, since only 11 subjects (15%) in our study showed minor or mild atypia at the entry of our study. Interestingly, Heimburgers folate and B-6 study[10] suggests that the observed effect in their study was primarily confined to the 8 subjects (11%) showing atypia on entry. On the other hand, Arnolds retinoid study[11] showed no effect in a trial with all subjects showing atypia at study entry.

It is tempting to interpret this study using a putative biomarker as indicating no influence of β-carotene on cancer risk. However, the predictive value of mild atypia for later development of lung cancer is only modest,[27] and other biomarker studies have shown effects of β-carotene. In this same study group, we recently demonstrated beneficial effects of β-carotene on DNA damage as reflected by micronuclei in sputum cells.[9] Likewise, Stich et al.[28,29] demonstrated beneficial effects of β-carotene on micronuclei in buccal mucosa cells in tobacco chewers, whereas Garewal[30] reported evidence for a beneficial effect on oral leukoplakia. Possibly, β-carotene does indeed effect human carcinogenesis, but not at all stages of carcinogenesis or at all cancer sites.

This study provides preliminary evidence that β-carotene has no influence on minor bronchial metaplastic changes as detected in sputum cytology. Moreover, the results indicate that the performance of future studies can be increased by repeated sampling or use of brushings to diminish variations in cytology, by increasing size and time span, and by pre-selection of subjects with more severe lesions. Therefore, cautious interpretation of the absence of an effect of β-carotene is warranted.

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Epilogue

This Chapter evaluates the contribution that the studies in this thesis have made to our scientific knowledge. In the first part of this Chapter methodological issues are considered to evaluate the validity of the studies for further interpretation. In the second part, the contribution of the studies to our understanding of biological mechanisms regarding carcinogenesis and the role of β-carotene is discussed. Implications and expectations for future research are discussed in the third part of this epilogue.

Validity issues

The studies discussed in the overview in Chapter 2 as well as the "new" studies presented in this thesis all have a common aim; to contribute to our knowledge of what is happening in the human body during the development of human cancer, and elucidating the role of β-carotene in this process. In interpreting studies, it is therefore good scientific practice to evaluate the validity of studies. Two aspects of validity can be distinguished. *Internal validity* refers to the question whether effects observed within a given study are really true, or whether these effects may have been distorted by flaws in the study design, conduct or analysis. *External validity* refers to the question whether the effects observed in a given study can be extrapolated or generalized to what is really happening in humans in the general population.[1,2] In this thesis, two types of studies are reported, i.e. observational studies (Chapter 3 & 4) as well as experimental studies (Chapter 5-8). For both study types, issues regarding validity are discussed below.

Validity of observational studies

The internal validity of observational or non-experimental studies may be hampered by three forms of bias.[1,2]

Selection bias refers to a distortion of the measured effect introduced by the procedures used to select the study population. For instance, in a case-control study on diet and cancer, selection bias can occur if controls having "healthy" eating habits are more willing to participate and thus are overrepresented in the study group. For the associations between biological markers that we report within the group of smokers, a selection bias seems highly improbable. In the comparisons between smokers and non-smokers in Chapter 3 and 4, however, selection bias could attribute to the observed differences in vitamin levels if health-consciousness was a stronger motivation to participate in non-smokers than in the smokers. However, the contrasts in vitamin levels between smokers and non-smokers in our study correspond closely to those in surveys that did not use smoking behaviour as a factor in subject selection.

Information bias refers to misclassification of study subjects, e.g. with regard to plasma levels of antioxidants or levels of intermediate endpoints. If this misclassification is random or non-differential all study groups that are being compared will be misclassified to an equal degree and associations will be attenuated. This is called bias towards the null. The misclassification can however also be differential and may lead to over- or underestimation in certain study groups. The phenomenon of information bias is illustrated by the observer and run variation in the SCE measure that we observed in Chapter 3. For the analyses within the group of smokers, results were not biased, since smokers were randomly divided over runs and observers. Nonsmokers, however, were differentially misclassified following their unequal distribution over runs and observers. In the comparison between smokers and nonsmokers multivariate adjustment was used to account for this information bias. Chapter 3 thus demonstrates that differential misclassification can either be avoided by study design or can be accounted for by adjustment in multivariate analyses. However, for a number of markers presented in this study, we also observed a large amount of error of yet unknown origin. Presumably, this error will be random and will therefore attenuate or obscure associations. Therefore, as we emphasize in Chapter 3, the absence of significant associations in the cross-sectional studies should only be interpreted with great caution as evidence of a complete absence of a relation in a biological sense.

Confounding bias is the phenomenon that the observed effect of a factor is mixed with the effect of a second factor. This bias occurs if the confounding factor is associated with both the factor of interest, as well as with the biological effect (e.g. disease) under study. In our study in Chapter 3, alcohol consumption could possibly be a confounder in the association between smoking and SCE levels, since smokers consume considerably more alcohol than non-smokers. However, the association between alcohol consumption and SCE level, if present, is very weak. Adjustment for differences in alcohol consumption therefore does not materially alter the difference in SCE levels between smokers and non-smokers. In Chapter 3 and 4, possible confounders have been discussed in detail. It seems improbable that the associations reported in these Chapters suffer from confounding bias since possible confounding factors were measured and accounted for in the analyses.

With regard to the external validity of an observational study, this clearly can only be good if the internal validity is also satisfactory. The external validity of a study can also be affected otherwise, since findings in a study group of a given age, sex, and race cannot necessarily be generalized to the entire human population. The effect of a factor may thus be different or not present in the entire human population. For the studies in Chapter 3 and 4, this cannot be ruled out, since the studies were carried out in a select group, i.e. male administrative volunteers. However, it seems improbable that the biological associations that we report will be different in other age groups or in females in the general population, or in people who were not willing to volunteer.

Validity of experimental studies

The concepts of information bias, confounding and selection bias can also be applied to experimental epidemiological studies, and can even be extended to other experimental work, e.g. animal or laboratory experiments. In experimental work, the influences of confounding, information and selection bias on internal validity can be eliminated by appropriate study design. In a randomized placebo controlled trial, as reported in this thesis, the placebo and treatment group can be expected to be comparable with regard to known or unknown confounding factors. To further ensure this comparability, pre-stratification on known confounding factors before randomization was used. Randomization and pre-stratification in trials can also be expected to eliminate possible differential information errors. In this case, however, the placebo and treatment group should be comparable with regard to the influence of measurement errors. Thus, in the studies in this thesis placebo and treatment group were equally divided over laboratory runs, measurements days, observers, or other sources of variation. With regard to nondifferential information errors, i.e. misclassification leading to bias towards the null, randomization does not offer a solution. Even properly randomized studies will fail to demonstrate associations if random error dilutes true associations and the study size is inadequate. In the design of our studies inevitable random error has been incorporated, since power calculations based on known random errors were performed to ensure an adequate study size. For the sputum cytology study in Chapter 8, however, there was no prior estimate of error or spontaneous variation. Chapter 8 therefore emphasizes that the limited study size and the large spontaneous variations warrant cautious interpretation of the lack of an effect.

Experiments such as those described in this thesis, in contrast with observational studies, can thus be designed to have an optimal internal validity. This optimal internal validity is an important reason why we chose to perform the experiments in this thesis. However, the external validity of experiments often poses problems. In animal or laboratory experiments, the external validity is hampered by uncertainties in extrapolation from *in vitro* to *in vivo* situations, between species, and from short term high dose exposures to more chronic and low dose exposures. In human experiments, uncertainties regarding external validity still exist since it is not feasible to perform a life-time lasting experiment in a free living group of humans completely representative of the general population. Studies on cancer incidence thus necessarily focus on relatively short term trials in often elderly high risk groups, and may only have a bearing upon events in the late stages of carcinogenesis. Studies using biomarkers for cancer risk, such as those presented in this thesis, do not have this limitation. Biomarker studies may thus be the only opportunity to obtain valid information in earlier stages of carcinogenesis in humans. However, the external validity is uncertain for other reasons, since the predictive value of the applied markers for cancer development has not been established. Also, in the studies that we report, findings in male heavy smokers are described that may not apply to the general population. The results described

in this thesis therefore cannot be directly translated to human cancer risk, given the uncertainties regarding external validity. However, the studies do contribute to our understanding of the biological truth in humans, since they provide us with otherwise not obtainable information on biological mechanisms as measured in humans. In combination with other scientific work such as animal experiments, observational studies and trials on cancer incidence, biomarker studies thus provide a more complete picture to allow for a scientific judgement on the role of β-carotene in cancer prevention.

Biological mechanisms

In the studies reported in this thesis, we tested several hypotheses regarding the biological mechanisms of human carcinogenesis and the involment of β-carotene in this process. These hypotheses and their verification or falsification are discussed in detail in each separate Chapter. In this Chapter, we will try to integrate these results into a single conceptual framework. Moreover, this integrated interpretation will allow us to consider the implication of the study results for the formulation of new hypotheses regarding carcinogenesis and β-carotene. In Chapter 2, we described the current concepts of human carcinogenesis as a multistage process involving multiple genetic and epigenetic events. This concept of carcinogenesis, as depicted in Chapter 2, figure 1, shows conceptual similarities with the framework for the classification of biomarkers, as given by the US National Research Council[3] (figure 1).

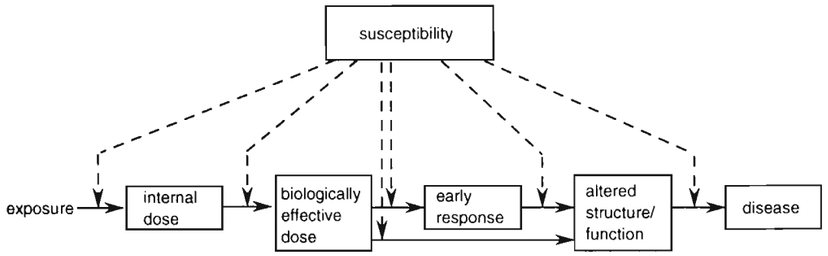

Figure 1. The relationship of biological markers to exposure and disease.

In analogy with this figure, it is tempting to categorize the biomarkers we used in this study as reflecting the different stages of carcinogenesis (initiation, promotion, conversion, progression) as given in Chapter 2, figure 1.[4] Following this analogy, SCE and micronuclei could be considered markers of early biological effects that reflect DNA damage; these markers would then be relevant in the initiation phase of carcinogenesis. Likewise,

measurements of immune response would bear relevance to the promotional stages of carcinogenesis, since immune surveillance may prevent the occurrence of altered cell structures that show immunogenic properties. Metaplastic changes in sputum then would reflect altered structures in preneoplastic lesions and would thus bear relevance to the late (conversion/progression) stages of carcinogenesis. This categorization of the biomarkers within the framework of carcinogenesis is conceptually attractive and may indeed be helpful for an integrated interpretation of the results in this thesis. It should be realised, however, that reality is more complex than our concepts. For instance, DNA damage is probably indeed involved in early initiation stages of carcinogenesis, but may also significantly contribute to the later stages of carcinogenesis.[4]

With regard to biological mechanisms, the results in this thesis seem to indicate that the two markers for DNA damage (micronuclei in sputum vs SCE in lymphocytes) may in fact reflect different biological phenomena. Firstly, because we did not observe a correlation between SCE levels and micronuclei counts. Secondly, because we did observe an association between the epoxide detoxifying enzyme GST-μ and SCEs, in contrast with no association for GST-μ and micronuclei. It may thus be hypothesized that epoxides are important in the induction of SCEs by cigarette smoke derived carcinogens, whereas this may not be the case for micronuclei. This hypothesis regarding the role of epoxides in SCE and micronuclei induction is also supported by the differential results with regard to the β-carotene supplement: we observed no effect upon SCE levels, as contrasted with a considerable reduction in micronuclei counts. Free radicals probably play an important role in several forms of DNA damage, but not in the *in vivo* production of epoxide intermediates nor in the reaction of epoxides with DNA.[5,6,7] Thus, if β-carotene functions as an antioxidant neutralizing free radicals, whereas SCEs are primarily induced by epoxides, this would explain the different response of micronuclei and SCEs after β-carotene supplements. Following the arguments given above, the different responses of SCEs and micronuclei after β-carotene also seems to support the hypothesis that β-carotene is effective as an antioxidant, and not as a pro-vitamin A. This notion is also supported by the unchanged plasma retinol levels during the trial. Results regarding buccal micronuclei as reported by Stich[8] also seem to support a non-retinol mediated effect of β-carotene since subjects in this latter trial had normal retinol levels, though retinol levels were not monitored during the trial. Likewise, Watson[9] reports effects of β-carotene on lymphocyte subsets, also without changes in plasma retinol levels. However, our study failed to confirm these results (Chapter 7), possibly because of differences in age between the study groups.

Apart from differences in molecular mechanisms contributing to SCEs versus micronuclei, the different effects of β-carotene may also be explained by site-specificity. Biomarker studies that have so far shown positive effects of β-carotene (e.g. oral leucoplakia[10] and micronuclei[8,11] all relate to tissues directly in contact with tobacco components. This may

measurements of immune response would bear relevance to the promotional stages of carcinogenesis, since immune surveillance may prevent the occurrence of altered cell structures that show immunogenic properties. Metaplastic changes in sputum then would reflect altered structures in preneoplastic lesions and would thus bear relevance to the late (conversion/progression) stages of carcinogenesis. This categorization of the biomarkers within the framework of carcinogenesis is conceptually attractive and may indeed be helpful for an integrated interpretation of the results in this thesis. It should be realised, however, that reality is more complex than our concepts. For instance, DNA damage is probably indeed involved in early initiation stages of carcinogenesis, but may also significantly contribute to the later stages of carcinogenesis.[4]

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also be considered to support an antioxidant mediated effect of β-carotene, since it is likely that the directly exposed (oral and tracheobronchial) tissues suffer most from oxidant stress. However, it is also possible that carcinogens from cigarette smoke induce localized retinol deficiencies that are more efficiently neutralized by (putative) β-carotene conversion at tissue level[12,13] than by supply of retinol from the circulation. In this respect, it is of interest to note that a similar theory on localized deficiency due to cigarette smoke exposure has recently been formulated with regard to folic acid.[14] Since we did not measure retinol levels in sputum cells, an effect of β-carotene on micronuclei through conversion to retinol following localized tissue deficiencies cannot be excluded. However, antioxidant effects of β-carotene are at this moment supported by ample experimental evidence,[15] whereas localized conversion to retinol remains to be established.

From the results for the micronuclei and SCE measures, we may thus conclude that β-carotene seems to protect against certain, but not all, forms of cigarette smoke induced DNA damage, especially in target tissue exposed to cigarette smoke components. This is in line with previous results for buccal micronuclei.[8,11] Following the previously discussed classification of biomarkers in the pathway of carcinogenesis, this may imply a protective effect of β-carotene in the early or initiation stages of carcinogenesis. Since we observed no influence of β-carotene on metaplastic changes in bronchial sputum cells, our results may thus be construed as indicating an effect of β-carotene primarily in these early phases. For several reasons, however, this interpretation must be cautiously viewed, and may not be fully warranted. Firstly, because we also observed an effect of β-carotene, though moderate, on aspects of immune functioning. The relevance of increased T cell mutagenesis for cancer immune surveillance remains uncertain, but these results may indicate an involvement of β-carotene in later stages of carcinogenesis through immune modulation. Secondly, it should be realized that DNA damage has been implied in cancer initiation, but may also contribute to later promotional stages of carcinogenesis (see Chapter 2). Finally, our metaplasia results do not provide a tendency for a beneficial effect, but the limited size of the trial and the large variations in sputum cytology warrant cautious interpretation of these metaplasia results.

The hypothesis that β-carotene is indeed primarily effective in the earlier stages of carcinogenesis has attractive aspects since it may, apart from site specificity, help to explain the recently reported lack of an effect of β-carotene in trials on cervical dysplasia[16] and second skin cancers.[17] On the other hand the preliminary evidence for effects on oral leucoplakia[10] may not correspond with early stage effectiveness. Further information to test this hypothesis can be expected from the ongoing trials in cancer incidence: if β-carotene is primarily effective in earlier stages, then an effect would only be detected after a longer follow-up. Indeed, the Physicians Health Study has recently been extended to more than 10 years-follow.[18] This illustrates that results and hypotheses from biomarker research can have important implications for design and conduct of future studies on disease incidence.

Implications for future research

Intermediate endpoint research

Biomarkers are increasingly being used in epidemiology to estimate exposure, susceptibility, early biological effects and pre-malignant disease stages.[19,20] Markers for biological effects and pre-malignant stages are of special interest, since they can be considered as "intermediate endpoints" for cancer risk. These intermediate endpoints will be distinguishable earlier and will be less rare than clinical malignancies. Therefore, intermediate endpoints may permit quicker, smaller and less costly investigations that also provide more insight in biological mechanisms.[21,22,23] Though the concept of intermediate endpoints is attractive, methodological and practical information on the application of such endpoints is relatively scarce. For application in future studies, we therefore discuss this information for the three putative intermediate endpoints used in this thesis; SCEs in lymphocytes, micronuclei in sputum cells, and metaplastic changes as observed in sputum cytology.

For the application of SCEs, it should be realized that they show only limited sensitivity towards variations in cigarette smoke exposure. Indeed, we observed only a 10-20% increase among smokers as compared with non-smokers (Chapter 3). Moreover, we observed considerable variation between laboratory runs and between two scorers, despite our efforts for standardization. In our study, almost 50% of the variation in SCEs was explained by variation between runs and observers. Also, we observed a very large variation over the 14-weeks period, that may have resulted from (a combination of) seasonal variation, diurnal variation, between run variation, as well as random variation of unknown origin. This biological and technical variability in the SCE measure implies that valid conclusions can only be drawn from carefully designed studies of sufficient size in which sources of variation are accounted for in design or analysis. The application of the SCE measure in epidemiological studies also has logistic constraints, since viable lymphocytes and a rather laborious cell culture process are necessary. Also possibilities for quality control are hampered by the inability to include a pool sample in each laboratory run. A better understanding of the sources of variation may lead to further standardization of the SCE determination, diminished random and non random error, and increased study power. Ideally, this standardization should also be applied over several laboratories to facilitate comparability of results of several studies.

For the micronuclei measurement, we observed no sensitivity towards the fluctuations in cigarette smoke exposure within our rather homogeneous group of heavy smokers. We did not have non-smoker data for comparisons since we found out that healthy nonsmokers do not spontaneously produce sputum. Other studies in a clinical setting have shown a 3-fold

increase in micronuclei in sputum[24] and bronchial brushings[25] in smokers compared to non-smokers. Despite inherent sampling variability, there was a moderate association (R = 0.3 - 0.4) between micronuclei counts in the same subjects sampled 14-weeks apart. Within-observer agreement was good, and there was no discernable between-run variation, possibly because the micronuclei parameter does not require *in vitro* cultivation of sampled cells, as contrasted with SCE. These results for the micronuclei determination indicate, that it may be a more attractive marker than SCEs in future studies. More so, because the logistics of the micronuclei marker are less complicated than the SCE parameter. Cells can be collected in fixative at participants homes and samples can be sent by mail. However, our data were obtained by scoring 3000 cells for each sample, which implies a major effort for a single observer. In the future, the prospect of automated scoring[26] therefore deserves further study. Also, future studies could be strengthened by repeated sputum sampling. Bronchial brushings are more site specific but have the drawback of being more invasive.

For the bronchial metaplasia marker, we again observed no association with cigarette consumption within our rather homogeneous group of heavy smokers. Agreement between and within observers was satisfactory, and comparable to agreements reported in other studies. We observed no association between sputum cytology scores in the same subjects measured 14-weeks apart, probably due to inherent sampling variability. This large within-person variability implies, that interpretations of sputum cytology scores are only useful at group level, and that interpretation of changes in individual scores over time is not warranted. A certain amount of observer and sampling variability, as in our study, seems inevitable for this marker. Future studies may decrease sampling variability by repeated sampling or by the use of bronchial brushing or biopsies. Also, future studies may benefit from a pre-screening to select subjects showing more severe stages of metaplasia on repeated sampling.

The intermediate endpoints described in this thesis are among those more frequently used.[27] For these markers, the biological and molecular mechanism underlying the marker are not exactly known. In terms of the potential of interpretation of results and contribution to understanding of mechanisms, markers at a more molecular level may prove useful in future studies on carcinogenesis. The GST-μ phenotype characterization reported in this thesis can be considered as a marker for susceptibility (see figure 1) at a molecular level. In this respect, recent developments in the measurement of specific DNA adducts,[28] oncogenes and tumor suppressor genes[29] and molecular markers for cellular differentiation and proliferation[23] may offer exciting opportunities for future molecular epidemiological studies. With regard to β-carotene and cancer risk in smokers, assessment of aromatic DNA adducts[30] or specific benzo[a]pyrene adducts[31] could be of interest. Also, assessment of 8-hydroxylation of the guanosine base could be a measure as an indication of *in vivo* oxidative DNA damage.[32] Since carotenoids have been reported to up regulate gap junction communication by stimulating connexin43 gene expression,[33] measurements of tissue levels

of this protein[34] could provide a molecular marker for regulation of cell proliferation. For cancer at other sites, expression of ras-oncogene[35] or accumulation of P53 tumor suppressor protein[36] have been suggested as intermediate markers for carcinogenesis. A final and very important issue in the future of intermediate endpoint research will clearly be the assessment of their validity in predicting cancer. As discussed in detail by Schatzkin,[21] this involves the incorporation of biomarkers in either prospective cohort studies or in intervention studies on cancer incidence.

Carotenoid and antioxidant research

The results in this thesis are a contribution to the ongoing research into the possible cancer preventive properties of carotenoids. With regard to β-carotene research, a number of desirable future epidemiologic studies that could be mentioned after performing our studies need not to be elaborated here, since several intervention studies on cancer incidence or intermediate endpoints are currently being conducted (see Chapter 2). Moreover, in Chapter 9.2 we have discussed several suggestions for intermediate endpoints at a molecular level that could be relevant to β-carotene research. Results from the ongoing intervention studies, especially with regard to cancer incidence, are eagerly awaited and can be expected to yield more definite answers regarding the putative cancer preventive potential of β-carotene. As we have discussed, the results described in this thesis may imply the need for a longer follow up in the trials on cancer incidence. Also, our studies may imply that an effect of β-carotene may be more readily discernable in smokers, being a high risk group with regard to DNA damage and cancer occurence.

If β-carotene does indeed prove to be beneficial in the ongoing trials, further laboratory study of the mechanisms of action will still be very meaningful for future possibilities for prevention of cancer. If the mechanism is primarily dependent upon conversion to retinol, this would imply that only carotenoids with pro-vitamin A activity will be beneficial. If, however, the mechanism of action (also) involves other, e.g. antioxidant, pathways, this would increase the scope to other potentially protective carotenoids that deserve further epidemiological and laboratory study. Moreover, the scope would then not be limited to carotenoids, but also to other antioxidant compounds (e.g. tocopherols, flavonoids and polyphenols) that are present in fruits and vegetables.[37,38] Future studies could then face the challenge to study not only individual antioxidant components, but also the effects of synergism and interaction between antioxidants. From the results of our studies, an antioxidant mediated pathway for β-carotene seems plausible, though it has not been proven. The further study of both nutritive and non-nutritive antioxidants in cancer prevention therefore remains a promising field for future research.

Conclusions

This thesis has evaluated a number of studies on cancer risk in humans using biomarkers with emphasis on the role of β-carotene. Regarding the application of biomarkers, the work in this thesis indicates that sources of variation in biomarker assessment need to be carefully incorporated into study design and analysis to allow internally valid conclusions to be drawn from biomarker studies. Moreover, logistic constraints may presently limit the applicability of a number of biomarkers in epidemiological study designs.

Though the predictive value of the biomarkers in this thesis is not established yet, these markers can provide information on biological mechanisms that would otherwise not be available from human studies. The data thus suggest, that increases in certain forms of DNA damage in GST-μ deficient smokers may explain the reported association between GST-μ deficiency and lung cancer risk. The results also indicate, that SCE and micronuclei, both markers of DNA damage, may in fact reflect different biological phenomena. With regard to β-carotene, the results suggest that β-carotene may indeed reduce cancer risk in humans by preventing cigarette smoke induced DNA damage. Also, β-carotene may enhance certain aspects of immune function. Moreover, the data are suggestive that β-carotene is effective per se, and not through conversion to retinol: this *supports an antioxidant mediated mechanism* for β-carotene. However, the data also indicate that β-carotene is not effective against all forms of cigarette smoke induced DNA damage and that β-carotene may not be effective in later stages of carcinogenesis as reflected by metaplasic changes. Our data thus substantiate and extend the implications from previous human studies, that β-carotene can affect human carcinogenesis, but not at all stages of carcinogenesis nor at all cancer sites.

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Summary

Carotenoids are yellow to red pigments that occur in vegetables and fruits. Carotenoids, especially β-carotene, have been hypothesized to decrease human cancer risk. The current state of knowledge regarding the cancer-preventive properties of carotenoids is summarized in Chapter 2. Epidemiologic studies have shown consistent inverse relations between intake of carotenoid-rich fruits and vegetables and risk of cancer at a number of common sites. For other sites, however, the evidence is not consistent. Mechanisms that could explain a protective action of carotenoids include conversion to retinol, antioxidant functions, immunomodulatory effects, as well as modification of carcinogen metabolism and cell-to-cell communication. Since observational epidemiology cannot resolve causal associations, human intervention studies on cancer incidence or on biomarkers related to cancer risk are necessary.

The hypothesis that β-carotene may decrease cancer risk is addressed in this thesis using an intervention trial in a high-risk group of heavy smokers. The trial evaluates the effect of β-carotene supplementation on several putative markers or intermediate endpoints for cancer risk. Two markers reflect DNA damage, which is a crucial event in carcinogenesis. These cytogenetic markers are sister chromatid exchanges (SCE) in peripheral lymphocytes, and micronuclei in sputum as expectorated from the respiratory tract. As indices for immune function lymphocyte subsets in peripheral blood as well as mitogen-induced lymphocyte proliferation response were used. Sputum cytology was used to evaluate bronchial metaplastic changes as a potential marker of later stages of carcinogenesis. Plasma levels of β-carotene, retinol and antioxidant vitamins were monitored during the trial, as well as plasma cotinine as a measure of smoking behaviour. In addition, we assessed deficiency of the detoxification enzyme glutathione S-transferase μ (GST-μ) as a potential marker of genetic susceptibility to cancer. All participants in the study were healthy male employees, who reported not to be exposed to xenobiotic chemicals and who volunteered for the study. Smokers were selected for the trial if they had smoked more than 15 cigarettes per day for over 2 years. For comparisons, a group of non-smokers (never smokers) was selected for a baseline measurement without participation in the β-carotene trial.

In Chapters 3 and 4, data from the baseline measurements are used to evaluate the application of a number of biomarkers in a cross-sectional study involving both the smokers (n = 156) and the non-smokers (n = 38). For the smokers group, this cross-sectional study also provides the baseline measurement of the intervention trial. As expected, smokers had higher levels of SCE (5.08 vs. 4.71 SCE per lymphocyte) and lower levels of the antioxidants vitamin C (36.6 vs. 54.8 μmol/l) and β-carotene (0.31 vs. 0.48 μmol/l), indicating increased oxidant stress. Within the group of smokers, the two measures of DNA damage, micronuclei and SCE, were not associated (R = −0.025) and showed only limited sensitivity to variations in cigarette smoke exposure. SCE were higher in GST-μ-deficient smokers (5.24 vs. 4.97 SCE per lymphocyte).

For micronuclei a similar GST-μ-related difference was not observed (4.3 vs. 4.9 micronuclei per 1000 cells).

The results of the β-carotene intervention are presented and discussed in Chapters 5 to 8. The 163 volunteers were prestratified by age, duration and quantity of smoking and randomly assigned to either 20 mg/day β-carotene (n = 80) or placebo treatment (n = 83) for 14-weeks. During the trial 13 smokers (6 placebo, 7 β-carotene) discontinued participation, whereas biomarkers were inadequate or not obtained in a varying number of participants. The β-carotene and placebo groups were comparable on all initial characteristics. Plasma levels of β-carotene in the treatment group increased 14-fold during the trial whereas plasma levels of vitamins or cotinine remained unchanged in both groups.

The trial showed no protective effect of β-carotene on DNA damage reflected by SCE in lymphocytes (Chapter 5). Changes in SCE counts were identical in the placebo group (n = 73) and the β-carotene group (n = 70), as were SCE values after treatment (4.37 vs. 4.24 SCE per lymphocyte). Micronuclei in sputum, on the other hand, were reduced after β-carotene treatment; after adjustment for initial values, the treatment group (n = 53) had 27% lower micronuclei counts than the placebo group (n = 61) at the end of the trial (Chapter 6). Lymphocyte subpopulations showed that the initial and final distribution over all B and T cell subsets was identical in both groups (21 β-carotene, 24 placebo) (Chapter 7). For *ex vivo* mitogen response induced by PHA, T cell proliferation was 12% higher in the β-carotene group (n = 24) then in the placebo group (n = 23). Bronchial metaplasia in sputum cytology was assessed in 33 β-carotene and 42 placebo subjects (Chapter 8). There was no decrease in metaplasia scores in the β-carotene group during the trial, and final metaplasia scores were similar in the placebo and the β-carotene group.

The epilogue (Chapter 9) evaluates the contribution that the studies in this thesis have made to our scientific knowledge. Sources of variation in biomarker assessment need to be carefully incorporated in study design and analysis to eliminate various sources of bias and thus to allow internally valid conclusions to be drawn. The randomized placebo-controlled intervention studies in this thesis fulfil these requirement, but the external validity is not certain since the predictive value of the biomarkers is not established yet. The markers can, however, provide information on biological mechanisms that would otherwise not be available from human studies.

In conclusion, the results indicate that increases in certain forms of DNA damage in GST-μ-deficient smokers may explain the reported association between GST-μ deficiency and lung cancer risk. The results also suggest that SCE and micronuclei, both markers of DNA damage, may in fact reflect different biological phenomena. With regard to β-carotene, the results indicate that β-carotene may indeed reduce cancer risk in humans by preventing cigarette smoke-induced DNA damage. Also, β-carotene may enhance certain aspects of immune

function. Moreover, the data suggest that β-carotene is effective *per se*, and not through conversion to retinol; this supports an antioxidant mediated mechanism for β-carotene. However, the data also indicate that β-carotene is not effective against all forms of cigarette smoke-induced DNA damage and that β-carotene may not be effective in later stages of carcinogenesis as reflected in metaplastic changes. Our data thus substantiate and extend the implications from previous human studies, that β-carotene can affect human carcinogenesis, but not at all stages of carcinogenesis nor at all cancer sites.

Samenvatting

Carotenoïden zijn gele, oranje en rode kleurstoffen die voorkomen in groenten en fruit. Er zijn aanwijzingen dat carotenoiden, en in het bijzonder β-caroteen, bij zouden kunnen dragen aan een verminderd risico op kanker. In dit proefschrift is het verband tussen β-caroteen en kankerrisico bestudeerd in een aantal epidemiologische onderzoeken bij gezonde vrijwilligers. Bij deze vrijwilligers zijn een aantal zogenaamde biomerkers gemeten die bedoeld zijn om een indruk te krijgen van het mogelijke kankerrisico.

In hoofdstuk 2 van het proefschrift wordt een overzicht gegeven van de huidige stand van de wetenschappelijke kennis op het gebied van carotenoiden en kanker. Epidemiologische onderzoeken hebben een consistent verband aangetoond tussen een hogere consumptie van groenten en fruit die rijk zijn aan carotenoiden en een lager risico op een aantal veel voorkomende vormen van kanker. Voor een aantal andere vormen van kanker zijn de aanwijzingen echter niet eenduidig. Een aantal werkingsmechanismen zouden een beschermende werking van carotenoiden kunnen verklaren, zoals omzetting in vitamine A, antioxidant werking, beïnvloeding van het immuunsysteem, een invloed op de ontgifting van kankerverwekkende stoffen en een werking op de communicatie tussen de lichaamscellen. Uit de studies tot nu toe kan echter nog geen oorzakelijk verband worden geconcludeerd. Hiervoor is interventie-onderzoek nodig, waarbij nagegaan wordt of consumptie van extra caroteen invloed heeft op het voorkomen van kanker of op biomerkers voor kankerrisico.

In dit proefschrift worden de resultaten beschreven van zo'n interventie-onderzoek met biomerkers bij een groep mensen met een hoog risico op kanker, namelijk rokers. Twee van de biomerkers die gemeten zijn geven een indruk van DNA beschadigingen, omdat schade aan DNA een belangrijke stap is in het ontstaan van kanker. De eerste merker voor DNA schade is het aantal sister chromatid exchanges (SCE), gemeten in bepaalde witte bloedcellen, de lymfocyten. De tweede merker voor DNA schade is het aantal micronucleï, gemeten in opgehoest longslijm. Als merker voor het immuunsysteem is in het bloed de verdeling van lymfocyten over subgroepen met een bepaalde functie bepaald. Daarnaast is de groeireactie van de lymfocyten in celkweek gemeten na toevoeging van stoffen die tot celdeling aanzetten. Als mogelijke merker voor de latere stadia in het kankerproces is sputum cytologie gebruikt; hierbij zijn metaplastische veranderingen in opgehoest longslijm beoordeeld. Behalve de merkers voor kankerrisico zijn een aantal andere biomerkers bepaald, waaronder bloedwaarden voor β-caroteen, vitamine A en antioxidant vitamines, en bloedwaarden voor cotinine als een maat voor het rookgedrag. Verder is in bloed de aan of afwezigheid van het ontgiftingsenzym glutathion S-transferase μ (GST-μ) bepaald; dit zou een merker kunnen zijn voor een erfelijk bepaalde gevoeligheid voor het krijgen van kanker.

Alle vrijwilligers voor het onderzoek waren gezonde mannelijke werknemers. De rokers in het interventie-onderzoek hadden allemaal gedurende twee jaar meer dan 15 sigaretten per dag

gerookt. Ter vergelijking is aan het begin van het onderzoek ook een groep mensen gemeten, die nooit gerookt hadden. Deze laatste groep heeft geen extra β-caroteen gekregen.

In hoofdstuk 3 en 4 worden de gegevens van de beginmeting bij 156 rokers en 38 niet-rokers gebruikt om de toepassing van biomerkers in een cross-sectioneel onderzoek te evalueren. Zoals verwacht, hadden rokers meer SCE's (5,08 vs. 4,71 SCE per lymfocyt) en lagere bloedwaarden voor de antioxidanten vitamine C (36,6 vs. 54,8 μmol/l) en β-caroteen (0,31 vs. 0,48 μmol/l). Dit geeft aan dat bij roken grotere oxidatie-stress bestaat. Binnen de groep rokers werd geen verband gevonden tussen de twee maten voor DNA schade, micronucleï en SCE's (R = −0,025), terwijl deze merkers slechts in beperkte mate gevoelig waren voor verschillen in blootstelling aan tabaksrook. De rokers zonder het enzym GST-μ hadden hogere SCE waarden (5,24 vs. 4,97 SCE per lymfocyt), maar niet duidelijk verschillende micronucleï waarden (4,3 vs. 4,9 micronucleï per 3000 cellen).

De resultaten van de interventie met β-caroteen worden besproken in hoofdstuk 5 t/m 8. De 163 rokers werden door het toeval toegewezen aan een groep die gedurende 14 weken 20 mg extra β-caroteen per dag kreeg (n = 80), of aan een placebogroep die gedurende dezelfde tijd een pil zonder werkzame stof kreeg (n = 83). Bij deze toewijzing werd rekening gehouden met de leeftijd en het rookgedrag van de rokers. Tijdens de studie hebben 13 deelnemers (6 placebo, 7 β-caroteen) zich teruggetrokken uit het onderzoek, terwijl bij een wisselend aantal personen niet alle biomerkers gemeten konden worden. De placebo en β-caroteen groep waren bij de aanvang van het onderzoek vergelijkbaar voor wat betreft bloedwaarden voor antioxidanten en vitamines. Tijdens de interventie werden de bloedwaarden in de β-caroteen groep 14 keer zo hoog, terwijl de bloedwaarden voor de antioxidanten en cotinine gelijk bleven in beide groepen.

In het onderzoek werd geen beschermend effect van β-caroteen gevonden op DNA schade in de vorm van SCE's (hoofdstuk 5). De veranderingen in SCE's tijdens het onderzoek waren gelijk in de placebo groep (n = 73) en de β-caroteen groep (n = 70), evenals de eindwaarden voor SCE's (4,37 vs. 4,24 SCE per lymfocyt). Voor de micronucleï werd wél een afname door β-caroteen gevonden; na correctie voor de beginwaarden had de β-caroteen groep (n = 53) 27% lagere eindwaarden dan de placebo groep (n = 61). De verdeling van lymfocyten over een groot aantal subgroepen was zowel bij de aanvang als aan het eind van de studie identiek in beide groepen (21 β-caroteen, 24 placebo) (hoofdstuk 7). Voor de groeireactie van lymfocyten na toediening van de stof phytohaemagglutinine werd een 12% hogere waarde gevonden in de β-caroteen groep (n = 24) in vergelijking met de placebo groep (n = 23). Metaplastische veranderingen in longslijm werden beoordeeld in 33 β-caroteen en 42 placebo deelnemers. Er was geen verbetering waarneembaar in de β-caroteen groep gedurende de interventie, en de uiteindelijke scores waren ook niet verschillend tussen de placebo en de β-caroteen groep.

In de epiloog van dit proefschrift (hoofdstuk 9) word besproken, wat de bijdrage is van de onderzoeksresultaten aan onze wetenschappelijke kennis. Er wordt geconcludeerd dat bij het opzetten en interpreteren van studies terdege rekening gehouden moet worden met allerlei redenen voor variaties in biomerker bepalingen, zodat de onderzoeksresultaten niet vertekend worden. In de dubbel-blinde, placebo gecontroleerde onderzoeksopzet in dit proefschrift lijkt zo'n vertekening van de onderzoeksresultaten onwaarschijnlijk. Bij het interpreteren van de resultaten moet echter wel bedacht worden dat we nog niet zeker weten of de biomerkers voor kankerrisico in het onderzoek inderdaad iets zeggen over het later krijgen van kanker. De biomerkers geven ons in elk geval wel een goed inzicht in de biologische werkingsmechanismen bij mensen.

De conclusies van dit proefschrift zijn allereerst dat een toename in bepaalde vormen van DNA schade bij rokers zonder het ontgiftings enzym GST-μ een verklaring kan zijn voor het verband in eerdere onderzoeken tussen erfelijk bepaalde afwezigheid van dit enzym en het optreden van longkanker. Verder lijken verschillende biologische mechanismen verantwoordelijk voor de twee merkers voor DNA schade, SCE's dan wel micronucleï. Voor wat betreft β-caroteen laten de onderzoeksresultaten zien, dat β-caroteen inderdaad het kankerrisico in mensen zou kunnen verlagen omdat β-caroteen bepaalde vormen van DNA schade door roken vermindert. Ook zou β-caroteen bepaalde aspecten van het immuunsysteem kunnen stimuleren. Bovendien suggereren de resultaten, dat β-caroteen deze effecten heeft zonder dat het omgezet wordt in vitamine A. Dit maakt het waarschijnlijker dat β-caroteen als antioxidant beschermend zou werken. De resultaten van het onderzoek geven tegelijkertijd echter ook aan, dat β-caroteen zeker niet werkt tegen alle vormen van DNA schade door roken, terwijl ook geen effecten aangetoond konden worden op metaplastische veranderingen die optreden in de latere fasen van het kankerproces. De resultaten van dit onderzoek zijn hiermee een onderbouwing en uitbreiding van de eerdere onderzoeken die wijzen op een invloed van β-caroteen op het proces van kanker, maar niet op alle vormen of stadia van kanker.

Nawoord

De bibliotheek van TNO Voeding in Zeist is gunstig gelegen met grote ramen op het zuiden en uitzicht op een park met oude beuken. Dit nodigt uit tot een langer verblijf dan strikt noodzakelijk, en de eerste ideeën voor de studies in dit proefschrift werden dan ook geboren in deze bibliotheek. Ideeën is één ding, en een helder projectvoorstel en dit ook nog uitvoeren is iets heel anders; hiervoor waren vruchtbare en soms verhitte discussies met Prof.Dr. Frans J. Kok onontbeerlijk. Frans, mijn dank voor jouw betrokkenheid en begeleiding bij 'ons' onderzoek. Ben je nu promotor in plaats van co-promotor omdat ik langzaam was, of omdat jij snel was? Ook Prof.Dr. Ruud J.J. Hermus, als promotor van het eerste uur, wil ik graag danken voor de steun die hij aan het onderzoek gegeven heeft.

Voor een epidemiologisch onderzoek zijn onderzoeksdeelnemers onmisbaar. Ik heb bewondering voor de vele vrijwilligers die nauwgezet supplementen hebben geslikt, en soms geduldig een wintersport kleur zonder hoogte-stage hebben doorstaan. Ook de instellingen die bereid waren hun deuren te openen ben ik veel dank verschuldigd. Bij het Centraal Belastinggebouw Utrecht was Fred van den Bosch altijd paraat om een onderzoeksruimte te regelen, terwijl bij het Energiebedrijf Utrecht Ton de Zwart een onaangenaam vroege start telkens weer mogelijk maakte. Bij AMEV gaven de bedrijfsartsen G.K.M. Maat en M.Th.J. Atjak hun steun, terwijl Trudy Hermans, Janna Middag en natuurlijk Alice van Zijl zorgden voor een grote hoeveelheid praktische en sociale ondersteuning. Alice, volgens mij deden een flink aantal vrijwilligers meer voor jou dan voor de wetenschap mee!

Bij de praktische uitvoering van een project wordt een onderzoeker geconfronteerd met fundamentele biologische beperkingen. Zo heeft ieder mens maar twee handen, terwijl veelal 90% van de hersencellen niet gebruikt wordt. Veel dank daarom aan Hanny Leezer, die deze beperkingen niet schijnt te hebben en die bovendien blijmoedig bestand is tegen de chaos die mijn actieve 10% weet te produceren. Hanny, ik weid verder niet uit, want we hebben maar twee pagina's voor het nawoord. Een ander rustpunt was natuurlijk Wim Gorgels, die nu veel meer ervaring met veldwerk heeft dan de doorsnee epidemioloog (sorry Wim).

Biomerkers betekent laboratoriumbepalingen. Tijdens het project heb ik precies één keer een pipet in m'n handen gehad, dus 'de uitbesteder' als bijnaam lijkt wel terecht. De micronucleï en SCE bepalingen door de Afdeling Biologische Toxicologie waren een tour de force voor rekening van Wilma Stenhuis, Truus Bruyntjes en Nico de Vogel. Het onmogelijke deden zij direct, op wonderen moest ik iets langer wachten, en tegenwoordig duiken ze niet meer weg als ik in de gang loop. Verder dank aan van André Tielemans en Bep van Tuijl, aan Jan Bogaards voor de GST-μ bepalingen en aan Jan Catsburg voor de 'difjes'. Bij de Afdeling Experimentele Biologie waren de vitaminebepalingen, een perfect systeem van opslag bij -80, en een mondsnoerend systeem van humor in goede handen bij Anneke Rademaker, Gonnie

Pieters, Jan van Schoonhoven, Jan Knijff, Freek Schrander en Frank van Schaik. Jolanda van Os en Lia Velthuizen waren bovendien altijd bereid om bloed te prikken (mooi werk hè, Lia, zo'n motor met zijspan). Mocht er na een bezoek aan EB nog een restje babbels over zijn dan was er altijd het Voedingslab met een prachtige eigen variatie op het mondsnoeren; veel dank aan Steven Spanhaak en Hillie Pellegrom voor de subsets, de stimuleringstesten, en de broodnodige uitleg hierbij.

Ondanks de vele laboratoria in Zeist zijn er ook nog essentiële bepalingen elders gedaan. I would like to thank Dr. Martin Jarvis of the Institute of Psychiatry in London for the cotinine assays. Voor de sputumcytologie is zeer prettig samengewerkt met de Afdeling Pathologie van de Katholieke Universiteit Nijmegen. Tiny Heynen deed de cytologische beoordelingen en heeft zo ervaren dat epidemiologie vooral veel betekent, terwijl Anniek van Aspert enthousiast een scoringsprotocol opstelde en de aanvullende scores deed. Mijn dank dat jullie ondanks het monnikenwerk toch telkens enthousiast waren als ik weer binnen kwam vallen!

Mijn allereerste idee van epidemiologie in een grijs verleden was dat het iets met computers was en dit bleek tijdens dit onderzoek weer waar. Voor hulp bij de data-invoer en verwerking dank aan Paul Duijzings, Evelien Aarnink en Ria Klokman, en aan Arthur van Aken voor de figuren. Verder natuurlijk dank aan Cor Kistemaker en Eric Schoen voor verkoelende statistische adviezen in tijden van cerebrale oververhitting.

Mocht de oplettende lezer nu denken dat deze promovendus niet veel zelf heeft gedaan, dan is dit het moment om te bekennen dat ik ook lang niet alles zelf bedacht heb. Voor de toxicologische advisering vanuit de afdeling Biologische Toxicologie zorgde Dr. Jan Wilmer tijdens de beginfasen van het project, terwijl Prof.Dr. Peter van Bladeren tijdens de latere fasen ook al te snel bleek om copromotor te blijven. Voor vitamine- en caroteen-expertise kon ik altijd terecht bij Dr. Jaap Schrijver van de afdeling Experimentele Biologie. Verder dank aan Dr. Theo Ockhuizen, destijds hoofd van de afdeling Voeding, voor de immunologische inbreng en de steun voor dit promotie-onderzoek. Prof.Dr. G.P. Vooijs van de afdeling Pathologie, KU Nijmegen was een onmisbaar adviseur op het gebied van sputumcytologie. Van Dr. Riekie de Vet van afdeling Epidemiologie, RL Maastricht, kreeg ik goede adviezen en haar proefschrift was tot voor kort het enige dat ik ooit helemaal gelezen heb.

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Geert van Poppel was born in Riethoven on 10 July 1960. After graduating from Gymnasium-β at the Rythovius College in Eersel, he started to study Human Nutrition at the Agricultural University in Wageningen in 1978. The MSc program was focused on nutrition (in particular nutritional epidemiology) and toxicology and included a project at the Tanzania Food and Nutrition Center in Dar Es Salaam. After obtaining his MSc degree in 1985, he worked at the International Course in Food Science and Nutrition in Wageningen. From 1985 to 1986, he worked as a research associate on an EC project at the Department of Human Nutrition, Agricultural University Wageningen. During this period, he also obtained a teachers' degree.

In 1986, he started working as a nutritional epidemiologist at the Department of Nutrition, TNO Nutrition and Food Research in Zeist. From 1986 to 1989 research was done within the framework of the Dutch Nutrition Surveillance program. During that time he wrote the grant proposal for the intervention study described in this thesis. In 1988, he attended the New England Epidemiology Summer Program at Boston/Medford, USA. From 1989 onward research activities include the studies in this thesis, several case-control studies on dietary factors and cardiovascular disease, and the role of non nutritive dietary compounds in disease prevention. Since 1993, he is head of the Epidemiology Section of the Department of Nutrition of TNO Nutrition and Food Research.

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Worteltjestaart

400 gram zelfrijzend bakmeel
1 eetlepel kaneel
1 theeleepel nootmuskaat
1 theelepel zout

3 kleine eieren
300 gram suiker
150 gram mayonaise

100 gram ananasstukjes
300 gram geraspte wortelen
75 gram walnoten
75 gram rozijnen

voor glazuur
100 gram roomkaas
50 gram poedersuiker
2 eetlepels water

Meng zelfrijzend bakmeel, kaneel, nootmuskaat en zout in een kom. Meng de eieren, suiker en mayonaise in een aparte kom en voeg dit aan het bloemmengsel toe. Kort mixen of roeren tot het deeg goed gemengd is. Roer vervolgens de ananas, wortelen, noten en rozijnen door het deeg en breng dit over in een ingevette tulbandvorm. Bakken in 60 minuten in een voorverwarmde oven op 190°C. Laat de taart afkoelen, keer op een schaal en glazuur de bovenkant met het mengsel van roomkaas, suiker en water.